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THE EFFECT OF DIET UPON THE ASCORBIC ACID CONTENT OF THE LIVER IN MICE.

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THIS investigation of the ascorbic acid content of the liver in mice is a continuation of earlier ones dealing with various strains of mice, and with the effects of polycyclic hydrocarbons and of pregnancy (Kennaway and Kennaway, 1944*a*, *b*; Kennaway, Kennaway and Warren, 1944).

METHODS.

Analyses of the ascorbic acid in the liver were carried out in 498 mice in all, chiefly female CBA and male C57, with a few of the opposite sexes of these two strains, and some of two lines (C_3H, DBA), of which large numbers were not available. The females of the pure lines DBA and C_3H show a very high incidence of mammary carcinoma, whereas in the lines CBA and C57 this form of cancer occurs seldom, if at all. The various diets given were made up, and named, as follows:

(*a*) *Food.*—The mixed diet given to the general stock of mice here (oats, bread, Millard Marsh diet, dog biscuits, a proprietary food "Rat Cake," cod-liver oil and marmite).

(*b*) *Diet.*—The vitamin-free diet prepared by The British Drug Houses, Ltd. (starch 63, casein 21, salt mixture 5·5, hardened vegetable oil 10·5 per cent). One hundred grammes was made up with 30 to 40 ml. water and 2 ml. cod-liver oil.

(*c*) *Yeast.*—80 g. of vitamin-free diet was mixed with 20 g. or 10 g. of yeast, and with water and cod-liver oil as in (*b*). The yeast used was at first the "B_1 rich" yeast, named B yeast in the tables (containing per g. approximately 900 μg. thiamin, 40 to 50 μg. riboflavin, and 200 to 300 μg. nicotinic acid), obtained from The Distillers Company, Ltd., Glasgow, and later the "Food Yeast" produced at the Chemical Research Laboratory, Teddington, for which we are indebted to Dr. A. C. Thaysen. Hence there were four "Yeast" diets, named B Yeast 10 or 20 g., and Food Yeast 10 or 20 g. The two strains of yeast appeared to act in the same way in so far as the present experiments were concerned.

(*d*) *Milk and skim milk.*—These were given *ad lib.* in the drinking vessels in place of water to C57 male mice only receiving "Diet" as in (*b*) above (Fig. 6). The skim milk was prepared here by centrifugation and removal of the cream.

(*e*) *Various compounds* (riboflavin, thiamin, methionine, xanthopterin, lysine, alloxan) were dissolved in water and mixed with "Diet" as in (*b*) above, with the object of showing whether any of these compounds can replace yeast or milk in raising the ascorbic acid content of the liver. The diet "Casein-riboflavin" was made up of Diet 100 g., vitamin and fat-free casein (British Drug Houses, Ltd.) 50 g., riboflavin 6 mg., water 60 ml.

Usually three batches of four mice each, of the same sex and approximately the same age and weight, were placed (*a*) on Diet, (*b*) on Yeast or Milk, and (*c*) on either Food or one of the above compounds, respectively. All the mice had been kept on "Food" before the experiment began. One mouse from each batch was killed on the same day after from 4 to 22 days of treatment (Figs. 1 to 8). The results on the whole indicated that the effects of the various regimes became evident after about 9 days, and hence the results shown in Tables II, III and IV are those obtained on and after the 9th day. The estimations were carried out by titration with 2 : 6-dichlorophenolindophenol in the manner described before (Kennaway, Kennaway and Warren, 1944*a*) with the use of a mechanical disintegrator. The results are shown in Tables II, III and IV, in the first two of which the data are arranged in descending order of the mean concentration of ascorbic acid in the liver. In considering any such results one must remember that the range of variation in the concentration of ascorbic acid in the liver may be round about 100 per cent, even in mice of the same sex and strain kept under the same conditions (Kennaway, Kennaway and Warren, 1944*a*). For this reason the standard errors of the mean concentrations of ascorbic acid have been calculated

TABLE I.—*Amounts of Compounds Added as Supplements to Diet.*

Compound.	Approximate amount mg. in 10 g. food as eaten.	Daily requirement of rats.*
Riboflavin, 4 mg.†	0·29	40 μg. for growth. 120 μg. for lactation.
,, 8 mg.†	0·57	—
,, casein, 4 mg.†	0·29	—
Riboflavin 6·4 mg.†	0·46	—
Thiamin	0·23	10 μg. for growth. 120 μg. for lactation.
Thiamin	0·23	—
Riboflavin, 8 mg.†	0·57	—
Methionine	18·00	20·5 mg. (Himsworth and Glynn, 1944).
Methionine	18·00	—
Xanthopterin	0·57	—
Lysine	114	The addition stated doubles the amount of lysine in diet.

* The data for which no other reference is given are taken from Griffith and Farris (1942).
† These are the amounts of riboflavin added to 100 g. diet, or diet + casein.

and are inserted in Tables II and III. The mean ascorbic acid concentrations in the livers of mice on the various regimens were compared with the mean concentration for the mice on the vitamin-free diet. The significance of these differences was estimated by the method of " t," and the probability " P " that the difference is significant is shown in Tables II and III (column K).

For the purposes of the discussion values of " P " less than 0·05 are regarded as indicating a significant difference.

The amounts of the compounds to be given in any such experiment present a difficult problem because in practice it may not be possible to test a large range of doses of each of any considerable number of compounds upon adequate numbers

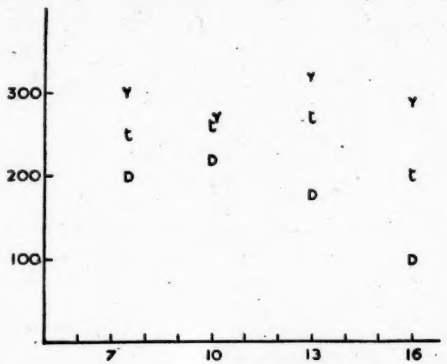

FIG. 1.—CBA female.

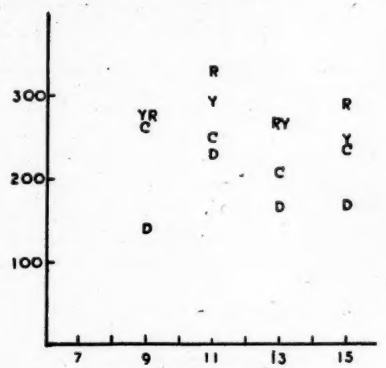

FIG. 2.—CBA female.

Legend to all Figures.

In Figs. 1 to 8, ordinates represent μg. ascorbic acid per g. liver. Abscissae represent days of the experiment. A, Alloxan; C, casein + riboflavin; D, Diet; F, Food; L, lysine; M, methionine; R, riboflavin; S, skim milk; T, thiamin; t, thiamin + riboflavin; X, xanthopterin; Y, yeast.

of mice of different sex and strain. As a rough guide we placed in 10 g. of moist food as eaten several times the daily amount required for a rat for lactation or growth (Table I). Such amounts may seem large, but our object was to see if any distinct results could be obtained.

The question whether the substance found in extracts of the livers of mice which reacts with 2 : 6-dichlorophenolindophenol is actually ascorbic acid was considered in an earlier paper from this laboratory (Kennaway, Kennaway and Warren, 1944*b*). The substance in question, and the additional amount of it which appears after intraperitoneal injections of 3 : 4-benzpyrene, was removed completely by vegetable marrow juice containing ascorbic acid oxidase; one may conclude therefore that this agent is ascorbic acid.

RESULTS.

CBA Females.

The results fall into two groups (Table II):

(1) The four series of mice receiving yeast show the highest mean concentration of ascorbic acid (mean 275), which is 47 per cent above that given by diet (187);

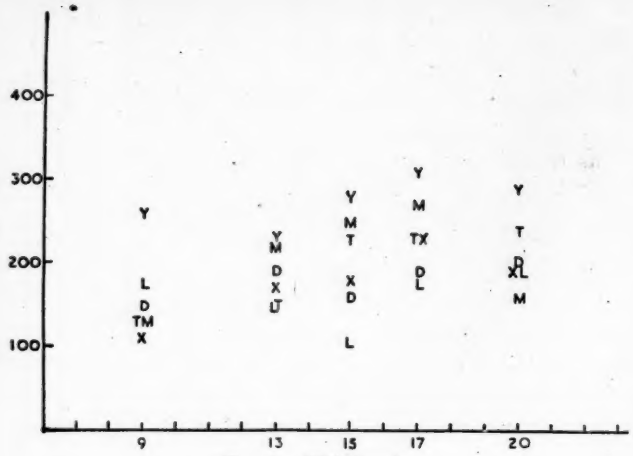

FIG. 3.—CBA female.

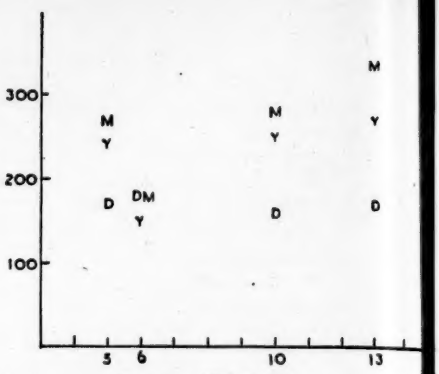

FIG. 4.—CBA female.

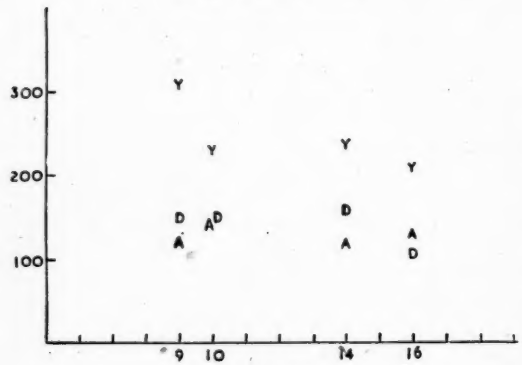

FIG. 5.—CBA female.

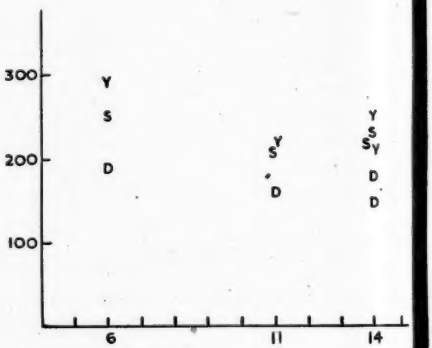

FIG. 6.—C57 male.

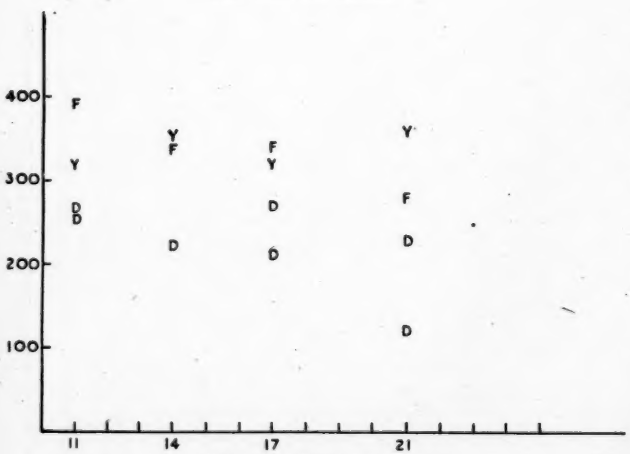

FIG. 7.—CBA male.

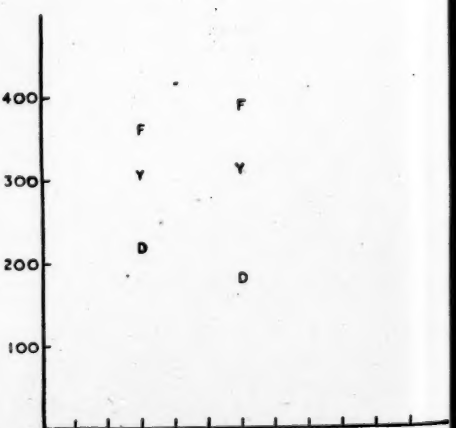

FIG. 8.—DBA female.

the four means differ very little (288, 268, 274, 269), which indicates that 10 g. yeast produces a concentration which is not increased by giving 20 g. (Figs. 1, 2, 3, 4 and 5). Yeast in these amounts raises the ascorbic acid to a level above that (239) given by the varied diet described under " Food " above.

(2) The addition to Diet of riboflavin, either alone or together with casein, in amounts of 4 mg. per 100 g. dry Diet, raises the ascorbic acid from the mean level given by Diet alone (187) to others (275, 256 mean 265), of which one is as high as the mean (275) given by yeast (Fig. 2). In this instance, then, riboflavin was, from the present point of view, a complete substitute for yeast. The combination casein + riboflavin was tried because this was found by Kensler, Sugiura, Young, Halter and Rhoads (1941) to be more effectual than either compound alone in preventing tumour formation in the liver by dimethylaminoazobenzene. Larger amounts of riboflavin (6·4 mg. or 8 mg. per 100 g.), either alone or together with thiamin (Fig. 1), or methionine, gave distinctly lower figures (234, 225, 212, mean 224), though this result depends upon fewer mice. Thiamin alone had no such effect (Fig. 3), but gave a result (188) not different from that given by Diet alone (187). Methionine alone (Figs. 3 and 4) gave a much higher result (255). Xanthopterin was tested on account of its structural relationship to riboflavin, which it does not, however, replace in any way as a supplement to Diet (Fig. 3). Lysine (Fig. 3) was chosen because it is a large constituent (22 to 25 per cent) of cytochrome C (Theorell and Akesson, 1941), which oxidizes ascorbic acid. Alloxan (Fig. 5) was tested because it is one of two compounds from which riboflavin can be synthesized ; it gave an extremely low mean result (131), which is, however, in accord with the unusually low figure (143) given by the Diet mice in this experiment. These negative results with thiamin, lysine, xanthopterin and alloxan have provided a valuable series of controls upon the positive results with riboflavin and methionine.

Column E in Table II shows that none of the animals receiving Diet, either with or without supplements, gained in weight, and all but one series lost weight, in some cases considerably. Supplements which maintained the ascorbic acid at a high level (riboflavin 4 mg., methionine) did not prevent the loss in weight.

The riboflavin present in the yeast given (40 to 50 μg. per g.) would provide from 0·5 to 1 mg. in 100 g. diet + yeast, whereas the riboflavin given as such was in larger amount (4 to 8 mg. in 100 g. ; Table I).

C57 Males.

These results are based upon smaller numbers of mice, and the differences observed are less distinct than was the case in the CBA female series (Table III).

In five series receiving yeast, milk, skim milk or food the mean concentrations of ascorbic acid lay within a fairly narrow range (215, 225, 225, 228, 233), mean 225, which is 30 per cent above that given by Diet (173). Milk or skim milk was therefore as effectual as yeast as a supplement to Diet, although the mice receiving milk lost weight (Fig. 6). Riboflavin, alone or together with casein or thiamin, gave results ranging from 218 to 195 ; the highest figure (218) is as high as that given by food yeast (215). The numbers of mice used are too small to establish any difference between the effects of the larger and smaller amounts of riboflavin.

TABLE II.—*Body Weight, Liver Weight and Ascorbic Acid of Liver of CBA Female Mice Receiving Various Diets and Supplements.*

CBA females (on or after the ninth day of the experiment).

Number of mice.	Treatment.	Body weight. Initial (on first day).	Body weight. Final.	Gain or loss.	Liver weight.	Liver weight percentage of body weight.	Ascorbic acid in liver. Total.	Ascorbic acid in liver. µg. per g. liver weight.	Ascorbic acid in liver. Standard error.	Ascorbic acid in liver. µg. per g. body weight.	Probability "P" that difference is significant.
A.	B.	C.	D.	E.	F.	G.	H.	I.		J.	K.
22	B yeast (20 g.)	21·1	22·0	+	1·32	5·95	375	288	± 6·3	17·0	<0·001
19	Food yeast (10 g.)	22·7	23·4	+	1·3	5·6	351	268	± 8·1	16·0	<0·001
14	B yeast (10 g.)	21·9	23·0	+	1·31	5·7	359	274	± 6·8 (275)	15·6	<0·001
8	Food yeast (20 g.)	22·1	24·0	+	1·34	5·5	377	269	± 9·8	15·2	<0·001
9	Riboflavin (4 mg.)*	22·3	20·2	−	1·06	5·2	291	275	± 11·8 (265)	14·5	<0·001
7	Casein-riboflavin (4 mg.)*	21·8	16·7	−	0·97	5·8	248	256	± 8·3	15·0	<0·001
8	Methionine	23·2	21·1	−	1·23	6·3	346	255	± 16;9	16·1	<0·001
7	Food	21·3	22·4	+	1·104	4·9	261	239	± 10·0	11·7	<0·001
6	Thiamin-riboflavin (6·4 mg.)*	21·2	21·4	+−	1·26	5·1	254	234	± 11·2	11·9	<0·001
3	Riboflavin (8 mg.)*	20·7	18·1	−	1·00	5·5	223	225	± 26·3 (224)	12·4	0·15
8	Methionine-riboflavin (8 mg.)*	22·4	21·0	−	1·08	5·15	230	212	± 16·4	10·9	0·15
8	Xanthopterin	21·0	18·6	−	1·08	5·8	222	202	± 20·3	11·7	0·5
68	Diet	22·3	18·7	−	1·03	5·5	196	187	± 5·6	10·3	..
8	Thiamin	21·3	17·7	−	0·99	5·6	184·5	188	± 14·7	10·5	>0·9
8	Lysine	22·0	18·7	−	0·98	5·0	171	177	± 13·8	9·0	0·5
4	Alloxan	21·9	19·9	−	1·06	5·5	141	131	± 8·6	7·1	<0·001
207			Mean gain 1·2 g. Mean loss 2·9 g.								

* These are the amounts of riboflavin added to 100 g. diet, or diet + casein.

TABLE III.—*Body Weight, Liver Weight and Ascorbic Acid of Liver of C57 Male Mice Receiving Various Diets and Supplements.*

C57 males (on or after the ninth day of the experiment).

Number of mice.	Treatment.	Body weight. Initial (on first day)	Body weight. Final.	Gain or loss.	Liver weight.	Liver weight percentage of body weight.	Ascorbic acid in liver. Total.	Ascorbic acid in liver. µg. per g. liver weight.	Ascorbic acid in liver. Standard error.	Ascorbic acid in liver. µg. per g. body weight.	Probability "P" that difference is significant.
A.	B.	C.	D.	E.	F.	G.	H.	I.		J.	K.
29	B yeast (20 g.)	25·3	25·7	+	1·48	5·9	338	228	± 4·6	13·0	<0·001
2	Food yeast (10 g.)	23·5	27·0	+	1·35	5·0	291	215	± 9·6	10·9	<0·001
7	Milk	25·4	22·8	−	1·16	5·2	271	233	± 10·4 (225)	12·0	<0·001
3	Skim milk	21·9	22·0	+−	1·07	5·3	240	225	± 4·6	11·9	<0·001
5	Food	25·7	26·9	+	1·47	5·5	332	225	± 23·6	12·5	<0·05
2	Riboflavin (4 mg.)*	23·2	17·9	−	0·92	5·1	199	218	± 17·0	11·2	<0·05
7	Riboflavin (8 mg.)*	27·6	23·2	−	1·2	5·1	238	200	± 7·8	10·3	<0·05
2	Casein-riboflavin (4 mg.)*	24·6	20·2	−	1·24	6·2	244	200	± 35·0	12·5	<0·05
2	Thiamin-riboflavin (6·4 mg.)*	23·2	18·6	−	1·05	5·7	205	195	± 13·0	11·0	>0·1
29	Diet	25·5	19·3	−	0·96	5·1	166	173	± 7·7	10·5	..
88			Mean gain 0·7 g. Mean loss 5·2 g.								

* These are the amounts of riboflavin added to 100 g. diet, or diet + casein.

Experiments on the Effect of Yeast, Food and Diet on Mice other than CBA Females and C57 Males.

These results are shown in Table IV, in which details of body weights and liver weights given in Tables II and III are omitted.

CBA male.—A single experiment on 15 mice on yeast, food, and Diet gave a clear result (Fig. 7 and Table IV).

C57 female.—An inadequate experiment in which 4 mice only were available after the 9th day gave an anomalous result (Table IV).

DBA male.—An adequate supply of Dilute Brown mice of either sex has not been available for these experiments. Only 6 males were available after the 9th day, and these gave the quite inconclusive result shown in Table IV.

DBA female.—Two experiments, of which one is shown in Fig. 8, on 8 mice in all gave clear and consistent results.

C_3H male.—Two experiments on 20 mice in all gave a fairly clear result (Table IV).

TABLE IV.—*The Effect of Yeast, Food and Diet, on Mice other than CBA Females and C57 Males.*

Strain.	Sex.	Number of mice.	Ninth day and subsequently. Ascorbic acid µg. per g. liver. Mean.
CBA	Male	4	Yeast 341
(Fig. 7)		4	Food 344
		7	Diet 227
C57	Female	2	Yeast 210
		2	Diet 233
DBA	Male	2	Yeast 281
		1	Food 402
		3	Diet 286
DBA	Female	3	Yeast 331
(Fig. 8)		2	Food 374
		3	Diet 226
C_3H	Male	10	Yeast 319
		3	Food 340
		7	Diet 261

The Concentration of Ascorbic Acid in the Liver in Different Pure Lines of Mice.

The results above obtained recently from mice receiving the laboratory diet ("Food") give an opportunity to compare the concentration of ascorbic aid in the liver in mice of the same strains examined in previous years (Kennaway, Kennaway and Warren, 1944*a*). Of course, close agreement cannot be expected when only very small numbers of mice are available, as in the DBA strain. But the later results (1945) confirm the earlier (1942) in showing (Table V) that the

concentration of ascorbic acid (1) is highest in the high cancer strains (DBA, C_3H) and in the males of the CBA strain (low cancer); (2) is considerably higher in the male than in the female CBA, and (3) is lowest in the C57 strain.

DISCUSSION.

The eight graphs shown are admittedly selected because they show the phenomenon in question especially clearly. All the experiments do not yield such satisfactory graphs, and many anomalous results are obtained. One or more

TABLE V.—*Comparison of the Concentration of Ascorbic Acid in the Liver in Different Pure Lines of Mice over an Interval of Three Years.*

Pure line.	Year.	Number of mice.	Ascorbic acid µg. per g. liver. Mean.
High cancer strains:			
DBA male	1942	28	344
,,	1945	1	402
DBA female	1942	4	467
,,	1945	2	374
C_3H male	1942	43	387
,,	1945	3	340
Low cancer strains:			
CBA male	1942	20	349
,,	1945	4	344
CBA female	1942	37	257
,,	1945	7	239
C57 male	1942	8	216
,,	1945	5	225

discrepant figures occur in almost every series (e.g. the value for the mouse receiving methionine on the 20th day in Fig. 3). But every single result obtained is included in the averages in Tables II, III and IV.

The phenomena in mice described here may be related to the failure of destruction of oestrogens in the liver of the rat on a vitamin-B-deficient diet described by Biskind and Biskind (1942).

After the above experiments were finished, Dr. Hoch-Ligeti suggested to us that the amount of ascorbic acid in the yeast given to the mice should be estimated. She made analyses accordingly by the ordinary method of titration, and Dr. F. L. Warren made estimations by the method of Roe and Kuether (1943). The results were as follows:

	Brewer's yeast.	Food yeast.
Indophenol titration	121 µg./g.	199 µg./g.
Roe and Kuether's method	38 µg./g.	153 µg./g.

By correspondence with The Distillers' Company and the Chemical Research Laboratory, Teddington, we learned that no other data were available as to the ascorbic acid content of these yeasts. The statement is met with in the text-

books that yeast contains no ascorbic acid. The amount of reducing substances found in yeast does not seem likely to account for the increase of the ascorbic acid content of mouse liver, because the feeding of the diet containing 20 g. of either of these two yeasts, differing widely in concentration of reducing substances, had no more effect on the mice than the feeding of 10 g.

SUMMARY.

The ascorbic acid content of the liver of mice (CBA male and female, C57 male, DBA female, C_3H male) receiving an ordinary diet falls when they are placed on a vitamin B free diet, and can be restored to the same or a higher level by a supplement of yeast. In C57 males, milk or skim milk has the same effect as yeast. Riboflavin, either alone, or together with casein, thiamin, or methionine, and methionine alone, have a similar if not so powerful supplementary action. Within certain limits, smaller amounts of riboflavin are more effectual than larger amounts. Thiamin, xanthopterin, lysine and alloxan gave negative results. Various discrepancies and anomalies occur which are described.

This investigation has been supported by generous grants, for which we express our thanks, from the British Empire Cancer Campaign, the Anna Fuller Fund and the Jane Coffin Childs Memorial Fund. We also wish to express our thanks to Dr. F. L. Warren and Miss M. M. Tipler, who have carried out a large part of the calculations of significance ; to Messrs. Organon Limited and to Dr. W. C. J. Ross for the xanthopterin used in these experiments and the preparation of solutions of it ; to Miss I. M. G. Shipton for drawing the graphs ; and to Miss M. I. Houchin for assistance in the analyses. We are indebted to Mr. P. J. Ewers for his care of the pure lines of mice used in these experiments.

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DETERMINATION OF COAGULASE AND ALPHA-HAEMOLYSIN PRODUCTION BY STAPHYLOCOCCI.

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WHEN studying the relationship of the coagulase, α-haemolysin, and hyaluronidase production of staphylococci (Schwabacher, Cunliffe, Williams and Harper, 1945), a number of strains were found that failed to produce an α-haemolysin, although they were definitely coagulase-positive. This observation conflicted with the earlier results of Cowan (1938) and of Gillespie, Devenish and Cowan (1939), who found all of their coagulase-positive strains to be α-toxigenic. Our discrepant results might have been due to the different techniques used for demonstrating coagulase and haemolysin, and accordingly we decided to investigate this possibility. In view of the widespread dependence on coagulase tests and of the great variety of techniques in use, it was thought wise to compare a number of the published methods, with the idea of finding which was the most reliable. At the same time we attempted to confirm earlier work on the slide-coagulase test (Cadness-Graves, Williams, Harper and Miles, 1943), and we compared three different tests for haemolysin production.

This paper reports, therefore, the testing of 152 strains of staphylococci for coagulase production by 11 tube methods and the slide method, and for haemolysin production by three different methods.

MATERIAL AND METHODS.

Source of strains.

The staphylococci were isolated from wound, nose, and skin cultures, and were usually tested within three weeks of isolation; for the most part they were selected at random from routine cultures, but a few were chosen because the coagulase test performed at the time of primary isolation gave an equivocal result. Of the 152 strains, 100 appeared, on the basis of the great majority of tests, to be coagulase-positive and are referred to as such; similarly 50 strains are regarded as coagulase-negative. The other two strains gave doubtful results and are discussed separately.

Source of plasma.

Oxalated plasma from the same two subjects was used for all the tests, usually within two days of bleeding; occasionally week-old plasma was used, but only for the slide tests.

Technique of coagulase tests.

The staphylococci were grown overnight in nutrient broth and on blood agar. The size of the inoculum, the dilution of plasma, and the diluent for each method

are shown in Table I. In this table the term " loopful " of culture from a solid medium means the amount of growth that is picked up when a 3 mm. " nichrome" wire loop is drawn across confluent growth for a distance of 1–1·5 cm. The " small inoculum " used for method 9 was about the quantity of growth represented by half a 1 mm. colony of a staphylococcus. We made a rough estimate of the size of the inocula in terms of viable organisms by a surface viable count (Miles and Misra, 1938; Davis, 1940); 0·1 c.c. of an overnight broth culture contained about 50×10^6 viable organisms; the loopful of solid culture contained about 600×10^6, and the small inoculum from solid culture 7×10^6 viable organisms.

In most of the methods we adhered closely to the proportions given in the original reports, quoted in Table I, though we did not always take the readings at the times advised by the authors. All the tubes, except those for method 1, which were held at room temperature throughout, were put in the incubator together and were examined after 3, 6, and 22 hours' incubation at 37° C. The tubes were then left at room temperature (15–20° C.) for 24 hours and examined again. Test-tubes 8–11 mm. in internal diameter were used for all the tests.

Reading of coagulase tests.

When examining the tubes, we assigned the result to one of five classes, according to the presence or absence of clotting or granularity, as follows:

1. Negative . . .	No clot, no granular or ropy growth.
2. Very doubtful . .	Slight granular or ropy growth, not broken up by shaking.
3. Doubtful . . .	Definitely granular.
4. Weak positive . .	Definite clot formation, but clot not extending throughout the fluid column.
5. Positive . . .	Good clot.

In routine practice we have regarded both 4 and 5 as positive, 1 as negative, and 2 and 3 as doubtful and therefore to be retested. The same grouping is adopted in this report.

Technique of slide coagulase tests.

For this test the method advocated by Cadness-Graves and others (1943) was used—that is, making a thick emulsion of the cocci in a drop of water on a slide, and adding to this a very small drop of plasma; in the present series the amount of plasma picked up by a straight wire was found to be sufficient. The reaction was regarded as positive only when clumping took place within about 5 seconds (see Fig. 1). We also tested all the strains by the slide method used by Salm (1945) and by Berger (1943), i.e. an attempt was made to emulsify a loopful of culture first in a drop of saline and then, if a smooth suspension resulted, in a drop of plasma.

Technique of haemolysin tests.

All the strains were tested by—

(1) The method described by Cowan (1938); the cocci were grown in 0·3 per cent nutrient agar in air containing 30 per cent carbon dioxide at 37° C. for

two days, the agar and cocci spun down, and 0·3 c.c. of the supernatant tested against 0·3 c.c. of a 2 per cent suspension of rabbit red cells. In addition a control, containing 10 units per c.c. α-antitoxin, was included for each strain.

(2) The method of McFarlan (1938), following the technique described by Gillespie and co-workers (1939); the organisms were grown in broth in air containing 30 per cent carbon dioxide at 37° C. for two days, and 0·3 c.c. of the broth was then tested against 0·3 c.c. of a 2 per cent suspension of rabbit red cells; an antitoxin control was included for each strain.

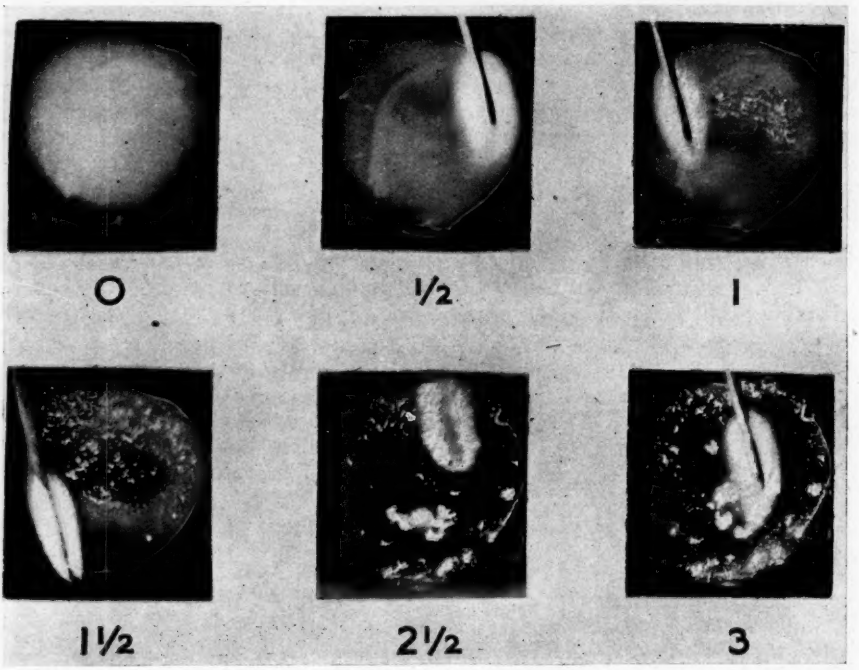

FIG. 1.—The slide clumping test—a good positive reaction (× 3). The figures indicate the interval, in seconds, after starting to stir the thick coccal emulsion with the wire wetted with neat plasma.

(3) On a sheep-blood agar plate, as suggested by Naidu (1934), Bryce and Rountree (1936), Kojima and Kodama (1939) and Christie and North (1941). Two plates were used for each set of tests, each containing 2 per cent sheep blood in nutrient agar, and one containing, in addition, two units of α-antitoxin per c.c. of medium. The blood supplied for Wassermann tests, containing 0·1 per cent formaldehyde, is satisfactory; but experiments carried out since this work was completed show that better results are obtained if washed sheep cells, rather than whole blood, are used (Munch-Petersen *et al.*, 1945). The plates were inoculated by stabbing the agar with a straight wire held perpendicular to the

surface, both test and control plates being inoculated without reloading the wire. The plates were incubated for two days at 37° C. in air containing 30 per cent carbon dioxide. Strains were considered α-toxigenic if they produced a hazy-edged zone of haemolysis on the test plate, while the zone of haemolysis on the antitoxin-containing plate was narrower and sharply defined; this sharply defined haemolysis does not seem to be due to either the α- or the β-haemolysin, and we intend to discuss its nature in a separate report. Strains were considered β-toxigenic if they produced a wide zone of darkening, or of partial haemolysis that was made almost or quite complete by subsequent refrigeration.

RESULTS.

Comparison of different tube coagulase methods.

In Table I are set out, for each different method, the number of strains that showed negative, doubtful, or positive reactions at the 6- and 22-hour readings. It should be noted in the first place that the 6-hour readings for method 9 would be expected to differ from the rest, since in this case the inoculum was small, and the growth at 6 hours was scanty. Considering the 22-hour readings, three methods gave a good clot with all the positive strains, namely, methods 2, 6 and 10; but, of these methods number 10 showed no fewer than 16 doubtful reactions among the 50 coagulase-negative strains—a proportion significantly higher ($d = 24$ per cent; $S.E._d = \pm 6{\cdot}3$ per cent) than the average of methods 2 and 6, which did not show a notable difference in this respect. Method 8 missed only one of the positives, and gave only five doubtful reactions among the negatives; since the difference of 1 per cent in the proportion of positive reactions is not significant, these methods, 2, 6 and 8, may all be regarded as satisfactory, though in fact methods 2 and 6 gave the quicker clotting. On the other hand, methods 1 and 11 are clearly unsatisfactory; 1 gave 34 doubtful and 8 negative reactions with the positive strains, and method 11 missed 13 of the positives, and showed 16 doubtful and 5 frankly positive reactions with strains that, by other tests, were negative.

It is interesting to compare the results given by methods 8 and 9, which differed only in size of inoculum. Method 9, with the smaller inoculum, gave doubtful reactions with nine and a negative reaction with one of the positive strains; while method 8, with a large inoculum, missed only one of the positives ($d = 9$ per cent; $S.E._d = \pm 3{\cdot}2$ per cent) and, incidentally, gave fewer doubtful reactions with the negative strains. We had previously used method 9 routinely, thinking that even if the proportions of culture and plasma were not optimal at the outset, growth of the cocci from the small inoculum would result in the optimum being reached at some time (see Duncan and Walker, 1942). The present results seem to show, however, that the formation of coagulase in the presence of the plasma does not result in such good clot formation as when culture and plasma are mixed in approximately optimal proportions in the first place. It is noteworthy that the proportion of plasma to culture is the same in methods 2, 6 and 10, namely, one part of plasma to one part of culture. The fact that this ratio gives the best results confirms the observations of Fisk (1940); the proportions used in methods 5 and 9 must be somewhat below the optimum. We cannot confirm Fisk's observation that clot formation was notably slow, or absent, when the plasma-broth was seeded with cocci from agar.

TABLE I.—*Results of Tube Coagulase Tests.*

Method number.	Reference.	Details of method.					Results (numbers of strains).											
		Plasma.			Inoculum.		100 coagulase-positive.						50 coagulase-negative.					
							At 6 hours.			At 22 hours.			At 6 hours.			At 22 hours.		
		Dilution.	Diluent.	Vol., c.c.	Culture.	Volume.	−	D.	+	−	D.	+	−	D.	+	−	D.	+
1	Chapman *et al.* (1938)	Neat	..	0·5	Solid	Loopful	45	31	24	8	34	58	49	1	0	40	10	0
2	Cowan (1938)	,,	..	0·5	Broth	0·5 c.c.	0	2	98	0	0	100	48	2	0	45	5	0
3	.. ..	1 : 2	Saline	0·5	Solid	Loopful	2	9	89	0	6	94	45	5	0	38	12	0
4	Fisher (1936) (method 3)	1 : 5	,,	0·5	,,	,,	1	13	86	0	11	89	49	1	0	44	6	0
5	Cruickshank (1937)	1 : 10	,,	1·0	Broth	,,	1	3	96	0	4	96	50	0	0	46	4	0
6*	Fisk (1940)	1 : 10	,,	1·0	,,	0·1 c.c.	0	0	100	0	0	100	49	1	0	47	3	0
7	. . . . .	1 : 10	,,	1·0	Solid emulsion in broth	0·1 c.c.	2	1	97	0	4	96	49	1	0	47	3	0
8	. . . . .	1 : 10	Broth	1·0	Solid	Loopful	1	4	95	1	0	99	48	2	0	45	5	0
9	. . . . .	1 : 10	,,	1·0	,,	Small inoculum	15	22	63	1	9	90	47	3	0	41	9	0
10	. . . . .	1 : 10	,,	1·0	Broth	0·1 c.c.	0	2	98	0	0	100	41	9	0	34	16	0
11	Gillespie *et al.* (1939)	1 : 2	Saline	0·3	,, 48 hours in CO_2	0·3 c.c.	2	13	85	2	11	87	44	6	0	29	16	5

* This is substantially the method recommended by Gillespie (1943).

D = Coagulation doubtful.

In general, successive readings up to 22 hours showed progression of clot formation, i.e. tubes showing a doubtful reaction at one reading were either doubtful or had become positive at the next. The 3-hour readings, which are omitted from Table I, usually showed less well-developed clots than the 6-hour, though methods 2 and 6 showed, respectively, 98 and 100 positives; method 8 showed only 85 positives. The 46-hour readings were usually the same as the 22-hour; only 19 of the 1100 tubes comprising the 11 tests of the 100 positive strains showed any progression, and of these 13 were tests with method 1; two were with each of methods 3, 9 and 11. Table II gives the number of coagulase-positive strains that showed dissolution of the clot at the 22-hour, compared with the 6-hour reading, and at the 46-hour compared with the 22-hour reading; few strains showed any dissolution up to 22 hours, although several showed varying degrees at 46 hours. Clearly the results, with methods other than No. 1, should not be read later than 22 hours.

It is evident that the different methods vary in the extent to which they give clear-cut results, and it is of interest to inquire whether doubtful reactions were also characteristic of particular strains. Of the 100 coagulase-positive strains 53 gave a doubtful or negative result by one or more methods at 22 hours; of these, 24 were doubtful or negative by method 1 only, and 32 were doubtful or negative by method 1 or 11. Excluding these two methods, which gave a high proportion of doubtful results in any case, 21 strains gave doubtful or negative results by one or more of the other 9 methods; 11 by one method only, 6 by two methods, 3 by three methods, and 1 by four methods. The property of giving false negative or doubtful results is not, therefore, one characterizing particular strains.

It is possible that a number of the doubtful results, and of the instances of breakdown of formed clot noted in Table II, resulted from excessive agitation in the early stages of clot formation, though care was taken to avoid shaking the tubes when making the 3- and 6-hour readings. It is also possible that some strains will only produce a good clot over a narrow range of plasma: culture ratios. Thus we observed one strain, not included in Table I, that was positive by the slide test and was α-toxigenic, and that gave positive results with methods 5 and 7, and doubtful results with methods 2, 3, 6, 8, 9 and 10. When tested

TABLE II.—*Dissolution of Clots with Increasing Time of Incubation.*

Method number.	Number of strains showing dissolution of clot.					
	At 22 hours compared with 6 hours.			At 46 hours compared with 22 hours.		
	Doubtful to negative.	Positive to doubtful.	Positive to negative.	Doubtful to negative.	Positive to doubtful.	Positive to negative.
1	6	0	0	0	1	0
2	0	0	0	0	5	0
3	0	1	0	0	2	0
4	0	2	0	1	1	0
5	0	4	0	0	4	0
6	0	0	0	0	2	1
7	0	1	0	0	6	3
8	0	0	0	0	0	0
9	0	0	0	0	2	0
10	0	0	0	0	0	0
11	2	4	0	1	14	4

with falling dilutions of plasma (Duncan and Walker, 1942) a clot was formed in the 1 : 2 dilution only. It is difficult to decide whether this strain should properly be regarded as coagulase-positive or negative. Another strain, whose classification is even more equivocal, gave a doubtful reaction on the slide, was non-toxigenic, gave positive results by methods 3, 7, 8, 9 and 11, and negative results by methods 1, 4 and by the method of Duncan and Walker. We tested, in all, 29 strains by this last method, but only in the case already referred to did we observe a definite clot over a narrow range only.

Slide coagulase tests.

Table III shows the results obtained with the slide coagulase tests. Not all the coagulase-positive strains gave a good positive slide reaction with either method. The fact that one of the coagulase-negative strains gave a slow positive reaction with the method of Cadness-Graves and others emphasizes the point made by those workers—that for the reaction to be regarded as positive clumping must take place very quickly after the addition of plasma (Fig. 1) ; slow reactions must always be confirmed by a tube test. Using this criterion, we have employed the slide test in our routine work over the past three years, and have not had any

TABLE III.—*Results of Slide Coagulase Tests by the Method of Cadness-Graves* et al. (C) *and of Salm* (S).

Result.	100 coagulase-positive strains.		50 coagulase-negative strains.	
	C.	S.	C.	S.
Negative	4	5	48	43
Auto-agglutinable	0	2	1	1
Doubtful	1	4	0	0
Weak or slow clumping	7	4	1	5
Positive	88	85	0	1

direct evidence of false positive reactions. As Table III shows, false negative reactions do occur, though they are not common. Indeed, of 2000 routine cultures, isolated during 1945, which were suspected of being *Staph. aureus*, 1624 were positive and 376 negative by the slide method ; tube tests were performed on the 376, and only 54 of them were positive. We have no doubt that the slide test is reliable if made with the precautions advised, namely, the use of a fairly thick suspension of cocci and a small quantity of plasma, and the requirement that a strain should only be regarded as positive if the clumping takes place within 5–6 seconds. The test should be relied upon only after some practice.

Table III also shows that the technique of Salm (1945) yields a similar proportion of clear positives, but a somewhat greater proportion of doubtful reactions among the coagulase-negative strains than the method of Cadness-Graves and others ($d = 10$ per cent ; $S.E._d = \pm 5{\cdot}7$ per cent).

Coagulase tests for routine use.

We may at this point summarize our opinion as to the best technique for the coagulase test, firstly for occasional strains isolated in a routine laboratory, and secondly for our own particular problem, namely, the testing of large numbers of strains from epidemiological surveys. For occasional tests, tube methods 2

or 6, or perhaps 8, would seem to be the most satisfactory. Both 2 and 6, however, require the use of two tubes and one pipette for each strain, and this raises a considerable supply problem when large numbers of tests are performed daily. For our own work we have, therefore, adopted the following routine: If enough pure growth is present on the direct plate, the strain is first tested by the slide method; if this is positive we accept the result. If the slide test is negative, and there is still sufficient growth, a tube test is performed by method 8; if there is insufficient growth for either of these, a colony is spread over a small segment of a blood agar plate, which is incubated overnight, and the resulting confluent growth tested by the slide method or tube method 8. The necessary tubes of 1 : 10 plasma-broth may be stored in the refrigerator for at least a week.

Plasma agar plate tests.

We have carried out a few tests with the plasma agar plate described by Penfold (1944) and by Reid and Jackson (1945). The first sample of plasma that we used was satisfactory, and we obtained good agreement with the tube tests. But, though we subsequently used a number of different samples of plasma, we did not find another that was satisfactory, so that an adequate test of the method was not practicable. It may be noted, however, that with some of our unsatisfactory samples of plasma, a few coagulase-negative strains gave well-marked haloes—a finding which emphasizes that the plasma should be tried with several strains of coagulase-negative as well as coagulase-positive organisms before use.

Haemolysin tests.

The results of the haemolysin tests on the 150 strains are shown in Table IV. Seven of the coagulase-positive strains failed to yield α-haemolysin demonstrable by either the sheep-blood plate or by MacFarlan's tube method; two of these strains yielded a β-haemolysin only. With Cowan's method, 12 of the coagulase-positive and 1 of the coagulase-negative strains showed haemolysis that was not neutralized by our α-antitoxin, even in a concentration of 100 units per c.c., or, in one case, 800 units per c.c. Cowan states that he attempted to neutralize the haemolysis only in a few cases, and he does not suggest that he had any failures. We have never observed unneutralizable haemolysis in tests by McFarlan's method, where the cocci are grown in broth rather than in sloppy agar, and the haemolysin produced in broth by the great majority of strains of *Staph. aureus* can be neutralized by 0·1 unit antitoxin per c.c., so that the observation that the haemolysis was not neutralized by the antitoxin in the tests mentioned

TABLE IV.—*Results of Haemolysin Tests.*

Method.	Number of strains producing α-haemolysin.	
	100 coagulase-positive strains.*	50 coagulase-negative strains.
Sheep-blood plate	93	0
McFarlan	93 (4 were weakly positive)	0
Cowan	83†	0

* Two strains produced β-haemolysin, demonstrable on plates and in tube tests with sheep red cells.
† See text, p. 79.

above suggests either that the addition of agar had increased the yield 1000-fold or that a different haemolysin was involved. In experiments with two strains of *Staph. aureus* we found that the addition of agar to broth resulted in no more than 4- and 8-fold increases respectively. As described elsewhere (Williams and Harper, in preparation), there is haemolysis demonstrable on sheep-blood agar plates containing α-antitoxin that is only neutralized by very large amounts of a potent α-antitoxic horse serum, and it is possible that this may be due to the same lysin as that which gave the unneutralizable haemolysis in our Cowan tests; this possibility is being investigated. Apart from the unneutralized haemolysis, no strain produced lysis when grown in sloppy agar (Cowan's method) that did not also do so in broth (McFarlan's method).

It is clear that we are still unable to confirm the findings of Cowan, and of Gillespie, Devenish and Cowan, that all coagulase-positive staphylococci are α-toxigenic. Six of our non-toxigenic strains were coagulase-positive by all 11 methods, and the other one was positive by 8 of the 11 methods. We have not, either in these tests or in other investigations, observed any of the strains described by MacFarlan (1938) that produce α-haemolysis but no coagulase.

SUMMARY.

One hundred and fifty-two strains of staphylococci were tested for coagulase production by 11 different tube methods and two slide methods, and for haemolysin production by two tube methods and the sheep-blood agar plate.

The most satisfactory tube coagulase techniques were those employing: (1) Equal volumes of plasma and broth culture containing 500×10^6 viable cocci per c.c.; (2) 1 c.c. of 1 : 10 plasma and 0·1 c.c. of broth culture of the same bacterial content; or (3) 1 c.c. of 1 : 10 plasma and a loopful of culture from a solid medium containing about 600×10^6 viable organisms. In the other methods used the percentage of false negative or doubtful reactions with coagulase-positive strains varied between 4 and 42 per cent. Only one method showed definite false positive reactions.

The slide clumping test for staphylococci was found to be satisfactory as a simple screening test, reliably identifying 88 per cent of coagulase-positive cocci and giving no false positive reactions, always provided that a thick emulsion of cocci was first made in water, a small volume of plasma added to it, and the reaction regarded as positive only if the clumping took place within five seconds.

Ninety-three of the 100 coagulase-positive strains produced α-haemolysin, and two showed only β-haemolysin. Sloppy agar appeared to be no better than broth for demonstrating the α-haemolysin.

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THE VIRUS CONTENT OF PLANTS SUFFERING FROM TOBACCO MOSAIC.

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THE method generally used for extracting plant viruses is to macerate infected leaves in a meat mincer and express the sap through cloth. In previous papers we (Bawden and Pirie, 1944, 1945) have shown that only a part of the virus present in the leaves is contained in the sap ; at least an equal amount is retained in the fibrous residues, from which it can be released in a soluble form by special treatments. Fine grinding in a triple roller mill has released several different viruses, including tobacco mosaic and tomato bushy stunt. With tomato bushy stunt virus, however, there was evidence that not all the virus in the fibre was released by this method, and that some was being destroyed (Bawden and Pirie, 1944). Incubation of the fibre with commercial trypsin preparations also released tomato bushy stunt virus, and more was obtained if the fibre was incubated with trypsin before milling than if the fibre was first milled and then incubated with trypsin. The experiments described below show that the fibrous residues of plants suffering from tobacco mosaic virus also contain more virus than can be set free by fine grinding, and that other enzymes are more effective than trypsin in bringing this virus into solution.

MATERIALS AND METHODS.

Most of the work has been done with tobacco (*Nicotiana tabacum*, var. White Burley) as a host plant, but tomato plants have also given similar results. The plants were grown in a heated glasshouse, but received no extra illumination, so that at different times of the year they varied greatly in their appearance and constitution. Nevertheless, the results of experiments done at different seasons have been reasonably constant; the same treatments have always been effective in releasing virus from the fibre, and the amount of virus in the fibre has always exceeded that in the sap. Similarly, the ratio of virus in the sap to that in the fibre is not greatly affected by the length of time the leaves have been infected, though the total amount of virus increases up to 4 weeks after infection. Leaves from 2 to 3 in. broad and 4 to 6 in. long were picked about a month after the plants were inoculated, and when they were showing well-developed symptoms. The midribs were cut out and discarded to avoid the presence of unnecessary fibre with a low virus content. The laminae were then minced, the sap extracted and the fibre washed and neutralized, as previously described (Bawden and Pirie, 1944). The washed fibre, squeezed as dry as possible by hand, has a water content of about 70 per cent, and forms the material for most of the extractions described. The technique used in passing the fibre through the roller mill was the same as that described previously.

The two main enzyme preparations used were from commercial trypsin and the alimentary tract of snails. Solutions of commercial trypsin were dialysed and clarified by low-speed centrifuging, and used at a final concentration of 2 g. of dry matter per litre. Although the effective constituent of this mixture has not been identified, similar results have been obtained with solutions of crystalline chymotrypsin. The mixture of snail enzymes was obtained from *Helix aspersa*. The snails were kept for some days without food, then killed with chloroform and removed from their shells. A slit was made in the left-hand side of the snail near the edge of the mantle, when the distended crop usually protrudes. It was pulled out gently with forceps, and the intestine and oesophagus were cut so that the crop falls into a beaker. When several crops were obtained, they were cut up roughly with scissors, diluted with water, so that there was 1 ml. of final suspension for each snail, and clarified by centrifuging. The fluid retains its activity, apparently unchanged, for some months at 0° C.

Samples of 0·5 g. (wet weight) washed fibre were mixed with 1 ml. of enzyme preparation and 0·3 ml. of 0·3 M pH 6·8 phosphate buffer and incubated for 48 hours at 37° C. with chloroform as disinfectant. Mixing is difficult because of the porridge-like consistency, and there are probably considerable variations in pH, but dilution with water or buffer slows down the reaction. During incubation the pH falls and it is necessary to add NaOH to keep the pH between 6 and 7, where there is the maximum effect with both trypsin and the snail enzymes. After incubation the volume of the mixture was made up to 10 ml. with water and clarified by centrifuging for 20 minutes at 3000 r.p.m. Before proceeding to any other treatment, the fibre was washed by allowing it to soak for about 20 minutes in 10 ml. of water and then centrifuging.

The virus content of the different extracts of infected leaves was estimated serologically, by determining the precipitation end-point of the fluids when titrated against tobacco mosaic virus antiserum. The sera used were all prepared by Dr. A. Kleczkowski, by the intravenous injection of rabbits with purified tobacco

mosaic virus; they give no reaction with any extracts of healthy plants. The precipitin tests were made at 50° C. by methods previously described, 1 ml. of antiserum at a dilution of 1 in 400 being added to 1 ml. lots of test fluids diluted serially with a factor of 1 in 2. The greatest dilution to give a precipitate visible to the naked eye after 3 hours' incubation with serum was taken to be the precipitation end-point.

The use of serological methods for estimating quantities of tobacco mosaic virus is complicated by the fact that the virus exists in different physical states, which behave differently in precipitin tests (Bawden and Pirie, 1945). These differences seem to depend on the degree of aggregation of the virus particles, and the same weight of virus in an aggregated state may give a precipitin titre more than four times as great as when unaggregated. Thus a change in the degree of aggregation can readily be interpreted as an increase in virus content. To reduce this uncertainty, before extracts were tested serologically, they were all subjected to treatments that cause considerable aggregation. This can be done by heating to 60° C. at pH 5·7, but with solutions of low virus content this method is not entirely reliable and incubation with trypsin is to be preferred. All extracts that had not been incubated with trypsin (or with phosphate and chloroform, which have a similar effect) in the course of their preparation were therefore incubated at 37° C. and pH 7 for 10 hours in the presence of 0·1 per cent commercial trypsin. After such treatment, the virus behaves like a typical bacterial flagellar antigen, precipitating rapidly with antiserum and giving a fluffy, open type of precipitate, with an end-point that is a satisfactory measure of the virus content of different extracts. There is, however, no information on the original state of aggregation of the virus in the different types of extracts, nor on the proportion of serologically active virus that is also infective.

Both trypsin and the mixture of substances in the snail enzyme are strong inhibitors of infectivity. When they are added to virus solutions there is an immediate reduction in the number of lesions produced. This effect is reversible, for infectivity is increased if the virus and inhibitors are separated by sedimenting the former in the ultracentrifuge or precipitating it with acid. The interpretation of infectivity tests on previously unaggregated virus, however, is rather uncertain because of the complication of aggregation; incubation with snail enzymes or trypsin usually leads to a relatively small, irreversible fall in infectivity, presumably because of the aggregation caused. Infectivity tests were made by the local lesion method on leaves of *Nicotiana glutinosa*, using a Latin square or similar design to reduce errors arising from variations in susceptibility between different leaves.

RESULTS.

Liberation of virus by milling and trypsin.

The release of tobacco mosaic virus from washed fibre by passage through the roller mill, and the ultracentrifugal fractionation of the resulting extract, has already been described (Bawden and Pirie, 1945). One passage through the mill does not give all the virus that can be liberated by milling, and further diminishing quantities can be obtained by successive millings and extractions with water. As with bushy stunt virus, there is evidence that the milling is either destroying some of the virus or converting it into some unextractable form.

TABLE I.—*The Effect of Varying the Sequence of Milling and Incubation with Trypsin on the Liberation of Tobacco Mosaic Virus.*

Sample 1.		Sample 2.		Sample 3.		Sample 4.	
Treatments.	Titre.	Treatments.	Titre.	Treatments.	Titre.	Treatments.	Titre.
Mill	1000	Mill	1000	Trypsin	500	Trypsin	500
,,	100	Trypsin	600	Mill	2000	,,	100
Trypsin	500	,,	100	Trypsin	1200	Mill	2000
,,	30	Mill	50	,,	20	Trypsin	1400
Trypsin	30	—	—	Mill	40	,,	40
						Mill	100
Totals	1660		1750		3760		4140

Each column deals with a separate sample from the same lot of washed fibre, which was subjected successively to the treatments listed. The serological titres given are the observed precipitation end-points of the extract multiplied by a factor to bring them to the standard ratio of 1 ml. of fluid from 1 g. wet weight of washed fibre.

This is shown in Table I, where the quantities of virus obtained from samples of one lot of fibre by four different sequences of extraction are compared. It will be seen that the greatest yield is obtained when the fibre is first incubated with trypsin and then milled, and that it is much reduced if the order of treatments is reversed. Also, the more extensive the milling, the less is the virus that can subsequently be recovered by trypsin.

To explain a similar loss of tomato bushy stunt virus we suggested that the virus was being attached to some constituent of the fibre during passage through the mill. As evidence in support of this, we showed that there is a similar loss of virus when purified preparations are mixed with fibre from healthy plants and the mixture milled, and that some of this virus could then be recovered by incubating the fibre with trypsin. We have now made similar experiments with tobacco mosaic virus; there is little or no absorption of virus when purified preparations are mixed with fibre from healthy plants, but there is considerable loss if the mixtures are milled. Only a small part of this lost virus can be recovered by incubating the milled fibre with trypsin or snail enzyme. It seems, therefore, either that the virus is being attached to some substrate unaffected by the enzymes, or that there is actual destruction of virus during the milling. Similarly, there are two possible interpretations of the increased liberation of the virus when milling follows incubation of infected fibre with trypsin. The enzymic digestion may destroy material with which the virus combines during milling, or it may so soften the fibre that there is less intense rubbing and local heating on passage through the mill. Digestion with the trypsin preparation does cause a very obvious softening, and after this treatment the fibre passes through the mill very rapidly. The digestion brings about one-third of the fibre into solution; most of this is protein, but carbohydrate, amounting to about 10 per cent of the dry weight of the fibre, also goes into solution. After the digestion, however, the fibre still contains some nitrogen, presumably in the form of protein.

In the mill extracts of infected fibre, some tobacco mosaic virus is in combination with chromoprotein. When the deep green extracts are clarified by freezing solid, thawing and centrifuging, the precipitate of chromoprotein contains virus that is not brought into solution on extraction with water. In this condition the virus combines with its antiserum, but is not precipitated as a result of such combination. The presence of this non-precipitating complex of virus and

chromoprotein can prevent the precipitation of free tobacco mosaic virus if this is not greatly aggregated. This combined virus can be released, as previously described for similar complexes of tomato bushy stunt virus (Bawden and Pirie, 1944), by hydrolysing the chromoprotein with trypsin. The virus is then obtained in an aggregated form and precipitates normally with antiserum. The precipitation end-points obtained from preparations made in this way suggest that about 3 per cent of the weight of the chromoprotein complex which separates from frozen mill extracts may be tobacco mosaic virus.

Liberation of virus by snail enzymes.

Even after preliminary digestion with trypsin there is no reason to suppose that milling has no deleterious effects, or that it releases all the virus contained in the fibre. Alternative methods have therefore been sought. More gentle methods of grinding, such as a Latapie mincer or a Waring Blendor or shaking with sand, were relatively ineffective in releasing virus. So were many enzyme preparations. Incubation with lysozyme from egg white, papain : HCN, pepsin, " Takadiastase," and another commercial extract made from moulds, " Luizyme," were all less effective than trypsin. The mixture of enzymes obtained from snails, however, was more effective both in disintegrating the leaf fibre and in liberating the virus. Incubation with this showed that the fibre contains more virus than can be set free even by trypsin and milling combined.

Liberation of all the virus cannot be brought about by one incubation with the snail enzymes. Increasing the time of incubation beyond 48 hours gives no increase of virus in the extract. Apparently not all the virus set free during the incubation comes into solution in the extract, for if the fibre is extracted again with water, or with a mixture of phosphate and the snail enzyme, there is often as much virus in this as in the first extract. On further extraction the amount of virus obtained falls, but more can be liberated if the fibre is again incubated with fresh enzyme. Usually three such incubations, each followed by one or two extractions of the fibre with water, are needed to liberate all the virus that can be obtained by this method. These phenomena are illustrated in Table II, where the precipitation end-points of successive extracts of infected fibre are

TABLE II.—*The Liberation of Virus from Washed Fibre by Incubation with Enzymes.*

Fibre 1.		Fibre 2.		Fibre 3.		Fibre 4.	
Treatments.	Titre.	Treatments.	Titre.	Treatments.	Titre.	Treatments.	Titre.
Snail	1000	Snail	5000	Snail	2000	Trypsin	320
Wash	1000	Wash	1300	Wash	600	Wash	80
,,	300	Snail	2000	Snail	1000	Trypsin	600
Snail	2500	Wash	1000	Wash	1000	Wash	40
Wash	2000	Snail	600	Snail	1000	Trypsin	30
,,	1300	Wash	300	Wash	600	Snail	1000
Snail	600	Trypsin	200	,,	100	,,	1000
Wash	160			Snail	160	Wash	1000
,,	80			Trypsin	100	Snail	1000
Snail	300					Wash	600
Wash	100						
,,	100						

Each column deals with a different lot of washed fibre, which we subjected successively to the treatments listed. The serological titres are the observed precipitation end-points of the extracts multiplied by a factor to bring them to the standard ratio of 1 ml. of fluid from 1 g. wet weight of fibre.

set out. It also shows that the snail enzymes liberate considerable amounts of virus that are not liberated by trypsin.

An estimate of the amount of virus liberated by different procedures can be obtained by totalling the precipitation end-points given by successive extracts. This has been done many times, and the amount obtained from the fibre by incubating with snail has always exceeded that present in the sap or that obtained from the fibre by other methods. A more precise comparison of the efficacy of different procedures can be made by mixing aliquots of successive extracts made from one sample of fibre, so that the final pool corresponds to a known weight of fibre, and then determining the quantity of virus obtained. This has been done both by comparing the precipitin titres of such pools, and by isolating the virus from them by ultracentrifugation and precipitation with acid and salts. Table III shows the results of two such experiments. From this it is clear that most virus is obtained from the fibre by snail enzyme and least by milling alone, whereas milling after previous incubation with trypsin is intermediate. The virus that is lost during milling is not recovered by subsequent incubation with snail enzyme, and the total amount liberated is much reduced if milling is the first treatment given to the fibre. Thus, if this loss is a consequence of attachment to some constituent of the fibre rather than of actual destruction of virus, the attachment is one that is apparently unaffected by snail enzymes and trypsin. Table III also shows that less than one-third of the total virus content of infected leaves is obtained in the sap.

We have shown previously (Bawden and Pirie, 1945) that the virus in mill extracts is, weight for weight, less infective than that in sap. By ultracentrifugation, the virus in both types of extract can be separated into fractions with widely different infectivities, but the mill extracts contain a greater proportion of virus with small particles and low infectivity. The significance of this is difficult to assess, because of the possibility that milling is inactivating the virus. The infectivity results in Table III, however, suggest that the virus obtained from the fibre is less infective than that found in the sap, for the purified preparation made from virus liberated either by snail or by trypsin are no more infective than virus purified from the mill extract. The whole problem is, however, com-

TABLE III.—*Yields and Infectivities of Tobacco Mosaic Virus Extracted by Different Methods.*

Type of extract.	Yield of virus in mg. per g. dry weight of washed fibre.	Infectivity. Average number of lesions per leaf at—	
		10^{-4}.	10^{-5}.
Sap	10·4	118	35
Milled fibre	22	72	14·5
Fibre incubated three times with snail enzymes	93	58	14
Virus from sap after incubation with trypsin	—	81	21·5
Sap	27	173	75
Fibre incubated three times with snail enzymes	72	89	17·5
Fibre incubated with trypsin, milled, then incubated with snail enzymes	41	91	18
Fibre milled, then incubated twice with snail enzymes	24	95	21

plicated because of the uncertainty as to the effects of aggregation on infectivity at high dilutions. It will be seen that the infectivity of the virus obtained from sap is reduced after still further aggregation by incubation with trypsin, but even then is still higher than that of virus liberated from the fibre. It seems likely, therefore, that virus particles of varying infectivity do, in fact, occur in the leaf, and that the virus contained in the sap has a higher proportion of highly infective material than that bound to the fibre.

Except for their aggregating effect, we have no reason to think that trypsin or the snail enzymes have any irreversible action on the infectivity of tobacco mosaic virus. There seems to be no destruction of the virus in any part of the digestive tract, for the faeces of snails that have fed on washed infected fibre are rich in infective virus. The enzymes responsible for the disintegration of the fibre and the liberation of the virus are largely confined to the crop, and extracts made by grinding the stomach wall or the hepatopancreas have little action. We have called the action enzymic, but the only evidence we have for this is that the activity of the preparations is destroyed by boiling. We have no information on the particular enzyme involved, and attempts to fractionate the preparations have failed. When ultracentrifuged at 70,000 r.c.f. some material sediments, but the activity remains in the supernatant fluid. None of the fractions separated with ammonium sulphate has been active, and although active material can be precipitated with alcohol or acetone, this leads to little or no fractionation.

The optimum pH for liberation lies between 6 and 7. This suggests that cellulase (or lichenase), the best known constituent of the mixture of enzymes contained by snails, is not responsible for the liberation of the virus, because cellulase has an optimum at pH 5·2 (Karrer, Joos and Straub, 1923). No certain conclusions can be drawn, however, for after an apparently ineffective incubation at pH 5·2, there is liberation of virus if a second extraction is made of the fibre at pH 7·0. A similar effect was noticed when fibre of tomato plants suffering from bushy stunt was incubated with trypsin (Bawden and Pirie, 1944), but it has not been studied in detail.

During digestion with the snail enzymes, from 60 to 70 per cent of the solid matter of the fibre goes into solution. The distinct leaf fragments disintegrate into a histologically formless mass in which nothing but a few cellulose fibres remain distinguishable. About 25 per cent of the residue after incubation is carbohydrate, as estimated by the orcin method (Pirie, 1936), and about 10 per cent is nitrogen. As the original fibre contains from 3·5 to 5 per cent nitrogen, two-thirds of the original nitrogen remains associated with the insoluble material. This is not unexpected, because the snail digestive fluid contains little or no protease. Most of this protein goes into solution on subsequent digestion with trypsin, but this treatment liberates no more virus.* This protein does not seem to impede the action of the snail enzymes, for they do not liberate any more virus or cause any more extensive digestion of fibre that has previously had its nitrogen content lowered by incubation with trypsin. Similarly, the action is not impeded by the presence of pectin and lipoids, for it is not facilitated if these are first removed by treating the fibre with 5 per cent ammonium oxalate solution or with a mixture of alcohol and ether. The addition of 10 parts of a mixture

* This is not true of all other viruses. Snail enzymes also liberate tomato bushy stunt virus, but only incompletely. Subsequent incubation with trypsin liberates some more of this virus after the residues have thoroughly incubated with snail enzymes.

of equal volumes of alcohol and ether to neutral solutions of purified tobacco mosaic virus precipitates the virus without causing any loss of serological activity, whereas in slightly acid conditions there is some denaturation. Precipitation from neutral solutions causes some loss of infectivity, though this is not readily interpretable, as the tests have not been done with solvents rigorously freed from peroxides, and loss of infectivity without loss of serological activity is known to result from oxidation.

DISCUSSION.

The two main points that call for discussion are the quantity of virus contained in infected leaves, and the reasons for variation in infectivity of virus preparations obtained in different ways. It is clear that the virus content of infected plants is much higher than was previously demonstrated, and that only a small proportion is obtained in the sap that has been the starting material for previous work. The virus that remains behind is an appreciable fraction of the insoluble protein of the infected leaf. The presence of considerable quantities of an abnormal protein in infected leaves was demonstrated by Martin, Balls and McKinney (1938, 1939), for they found an increase in the leaf nitrogen undigested by trypsin. After boiling the tissue this extra nitrogen became digestible. They pointed out that this behaviour was similar to that of the virus, and suggested that their figures set upper limits to the total amount of virus in the leaf. Depending on the susceptibility of the variety of tobacco used and on the duration of infection, they found from 16 to 170 mg. of this type of protein per g. dry matter in the leaf. Although for many reasons our results are not directly comparable with theirs, the yields of virus that we have obtained by actual isolation agree well with the middle of the range given by Martin *et al.* This is clear from Table III, which shows that 7·2–9·3 per cent of the total dry weight of the fibre can be isolated as virus after incubation with snail enzymes. As the virus has a nitrogen content of 16·6 per cent, these two figures correspond respectively to 1·2 and 1·5 per cent of nitrogen, or approximately one-third of the total nitrogen that remains in the washed fibre. In addition to this there is the virus in the sap, and the combined yield is about 100 mg. per g. of original leaf dry matter.

The occurrence of so large a percentage of the total nitrogen of the infected leaf in the form of an abnormal protein raises several interesting metabolic questions. We have made no systematic comparisons of the ratio of virus nitrogen to total nitrogen in the fibre during the course of infection, but the nitrogen content of healthy and infected plants has been studied by other workers. Stanley (1937*a*, *b*) stated that the total nitrogen of the plant was increased by infection, but this is not borne out by his data. His published figures show a slight increase in the percentage of nitrogen in the washed residue from minced leaves, but the infection caused such stunting of the plants that the weight of the residue was greatly reduced. As a result, healthy plants averaged 1·3 g. of nitrogen in this fraction and infected ones only 0·7. The protein and non-protein nitrogen that remained soluble after the addition of dipotassium phosphate* increased from 0·19 to 0·20 g. The normal leaf protein that is precipitated by phosphate was presumably included in the leaf residue, but this is not stated

* In this, as in many other papers published between 1937 and 1939, Stanley says he used disodium hydrogen phosphate, but his statements on the manner of use make it probable that the potassium salt was, in fact, used.

explicitly. Martin, Balls and McKinney (1939) and Takahashi (1941) likewise found a slight increase in the percentage of nitrogen in the infected leaf; in neither paper is the total dry weight of the leaf given, so that there is no evidence for an increase in nitrogen rather than a decrease in the other leaf components. It seems likely that the virus is synthesized at the expense of some normal leaf protein, and there is evidence for this. Martin *et al.* (1939) find that during the first few days of infection the increase in virus is accompanied by a comparable decrease in normal protein, and under conditions of nitrogen starvation Woods and DuBuy (1941) state that the infected leaf makes virus instead of normal chromoprotein. Similarly, Takahashi (1941) finds that the synthesis of virus proceeds in detached leaves kept in the dark when normal protein is autolyzing.

It is obvious that "normal leaf protein" is a mixture of very many proteins, and there is no reason to think that even the chromoprotein is a chemical individual. The multiplication of the virus is unlikely to affect all of these proteins similarly, so that conclusions drawn from gross chemical analysis may be equivocal. This is illustrated by the amino-acid analyses published by Lugg and Best (1945). They point out that tobacco mosaic virus and normal leaf protein differ in their amino-acid composition, and they give figures for the amide nitrogen, tyrosine, tryptophan, cystine and methionine respectively, in whole leaves, the soluble protein and the residue of healthy and infected plants. The tryptophan and methionine figures are the most significant, because the residue from healthy leaves has 1·9 and 1·4 per cent of its nitrogen in these forms, whereas the virus has 2·9 and 0. The figures for the infected residue are 1·7 and 1·1. If the virus in their leaf residue is simply added to the normal leaf protein, or replaces part of all the components equally, their methionine value is reasonable, but their tryptophan value is too low. The most logical interpretation of the results obtained by Lugg and Best is that virus infection specifically depresses the synthesis of components of the normal protein mixture that are richest in tryptophan.

We have already shown that extracts of infected leaves made in different ways contain virus with different infectivities (Bawden and Pirie, 1945). Because of the suspected deleterious effect of milling, and the possibility that it might be destroying the infectivity of some virus without destroying serological activity, the significance of these differences could not be assessed. However, as the virus in sap as well as that in mill extracts could be separated into fractions with widely different infectivities, it seemed probable that virus particles with different abilities to cause infection actually occur within the infected plant and are not artefacts. The work with the snail enzymes supports this view. There is no reason to believe that these enzymes have any destructive action on the virus, although they do inhibit infectivity and cause aggregation. The fact that the virus obtained from the fibre by incubation with snail enzymes is less infective than that from sap, even after the latter has been incubated with trypsin (Table III), suggests that there is a real difference between them. How to interpret these differences is not at all clear. Our methods of testing infectivity are reasonably good for comparing the relative infectivities of different virus preparations, but there is no method of measuring the amount of infective virus in any one preparation. To get infection with the most active preparations that we have made, even when transmission is facilitated by the use of abrasives, about 10^{-10} g. must be used, a weight equivalent to at least 10^7 virus particles. Many reasons can be advanced to explain this: There is probably considerable loss of

inoculum on the surface of the leaves, and it may be that a large number of virus particles are needed to cause infection. It is, however, equally possible that a single infective virus particle can cause infection, and that most of the particles are not infective. From the fractionations we have carried out on sap, it is clear that all previous preparations must have contained mixtures of particles with different degrees of infectivity; it is probable that in our most infective fractions this is also true.

In measuring infectivity we are merely assessing the ability of virus particles to establish themselves, and initiate the chain of events that leads to virus multiplication and the development of symptoms. It is generally assumed that all virus particles that have not been inactivated are capable of causing infection, but there is no positive evidence for this, and it is possible that there are particles unable to do so which nevertheless may have other activities in the cells in which they are produced. The systemic infection of uninoculated cells is brought about by the movement of virus particles from the inoculated cells. This mobile virus is likely to be that occurring in solution in the cell sap rather than that tied to the fibre in insoluble forms, and it is perhaps significant that, although this is a minor part of the total virus, it is the most infective.

Our results provide no positive evidence as to the manner in which the virus is held by the fibre. The methods we have used may liberate it either because they destroy substances with which the virus was combined to give insoluble complexes, or because they disrupt structural elements in the leaf so that the relatively large virus particles can more easily pass into the extracting fluids. There is also no evidence about the site of virus multiplication, for it is equally possible that the virus in the sap is derived by the gradual solution of originally anchored virus, as that originally soluble virus from the sap became anchored to the fibre as infection proceeds.

SUMMARY.

Virus can be released from the leaf residues of plants suffering from tobacco mosaic by fine grinding, or by incubation with either trypsin or the mixture of enzymes from snails' crops. Grinding causes loss of virus, and more virus is obtained if incubation with trypsin precedes grinding than if the operations are reversed. Most virus is released by incubation with the snail enzymes. Successive incubations are needed to release all the virus from the residues; this may amount to one-third of the total insoluble nitrogen of the leaf. The total virus in the leaves accounts for 10 per cent of their dry matter; less than a third of the virus is obtained in the sap, but this is the more infective.

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EFFECT OF PENICILLIN COMBINED WITH NEOARSPHENAMINE ON THE LESIONS AND SEROLOGICAL TITRE OF EXPERIMENTAL SYPHILIS.

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In a previous communication (Selbie and Simon, 1944) a comparison was made between the effects of a short course of penicillin and a single dose of neoarsphenamine on *Treponema pallidum* infection in the rabbit. Whereas the immediate effect of penicillin in rabbit syphilis was comparable to that of neoarsphenamine in the rapid disappearance of spirochaetes and diminution of the local lesion, the tendency to relapse was much greater with penicillin, even in relatively high doses. On the basis of these findings it was suggested that relapses might occur in human cases of syphilis treated with apparently adequate doses of penicillin, but even if such relapses were frequent, penicillin would have its place as a valuable adjuvant to other methods of treatment. An example of such a use for penicillin would be in supplementing arsenical treatment, whereby it might be possible to reduce the dose of the arsenical drug and its related toxic effects, and at the same time obtain therapeutic results comparable to those following full arsenical treatment. A further series of experiments was therefore designed to test the effectiveness of penicillin as an adjuvant to neoarsphenamine in the treatment of rabbit syphilis.

MATERIAL AND METHODS.

The strain of *Treponema pallidum* (virus Truffi) and the general methods for following the results of treatment were the same as those previously described (Selbie and Simon, 1944). In addition, serological tests were made at intervals before and after treatment. For this purpose a precipitation reaction was chosen because of the difficulty of allowing for the varying anticomplementary action of rabbit sera in the Wassermann reaction. In the choice of a precipitation test the sigma reaction was preferred to the Kahn because of the greater ease in reading and measuring the results. The technique used and the method of expressing the serological titre were those described by Dreyer and Ward (1923). The batch of antigen which was used throughout these experiments was also used for testing clinical sera, and proved highly sensitive in both the experimental and clinical series of tests. The sigma reaction also appeared highly specific in that no positive reactions were obtained with the sera of 20 of the rabbits that were tested before inoculation with *T. pallidum*.

In the therapeutic experiments shown in Fig. 1, each comprising four rabbits of the same passage treated simultaneously, treatment was given at different intervals after inoculation, but, as will be shown later, only in Expt. 3, where treatment was delayed to the 80th day, did the development of resistance in the

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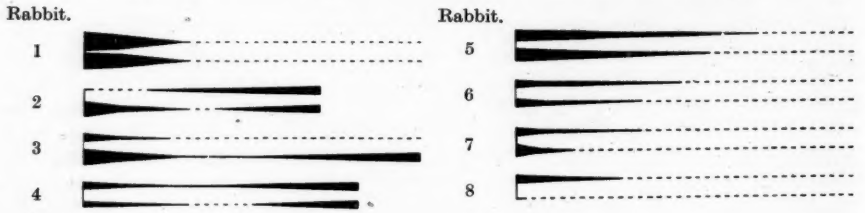
FIG. 1.—Effect of penicillin and neoarsphenamine on the lesions of rabbit syphilis.

For each rabbit the syphilitic lesions in the right and left testicles are represented by an upper and lower line respectively. The size of the lesion is represented by the width of the line, and when it is not palpable it is indicated by an interrupted line. Thus, in Rabbit 1 the right and left lesions had long diameters of 4·0 and 3·6 cm. at the commencement of treatment, and then diminished in size until they were no longer palpable at the 21st day. *Expt.* 1 *and* 2: Effect of a single dose of neoarsphenamine. *Expt.* 3 *to* 6: Effect of a single dose of neoarsphenamine combined with penicillin given in three 3-hourly doses. The total penicillin dosage per kg. was the same in all four experiments, but varied from rabbit to rabbit in each experiment, the dosage being: First rabbit (No. 9, 13, 17, 21), 30,000 units; second rabbit (No. 10, 14, 18, 22), 15,000 units; third rabbit (No. 11, 15, 19, 23), 9000 units; fourth rabbit (No. 12, 16, 20, 24), 4500 units.

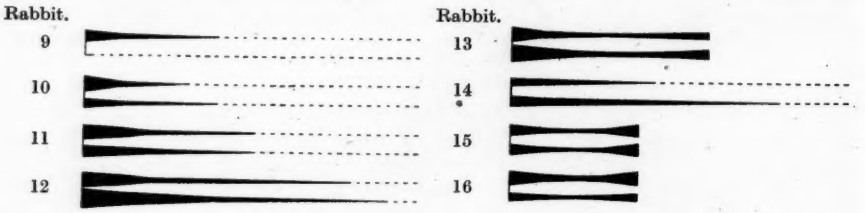
Expt. 1.—Neoarsphenamine, 10 mg. per kg., 52 days after inoculation.

Expt. 2.—Neoarsphenamine, 20 mg. per kg., 42 days after inoculation.



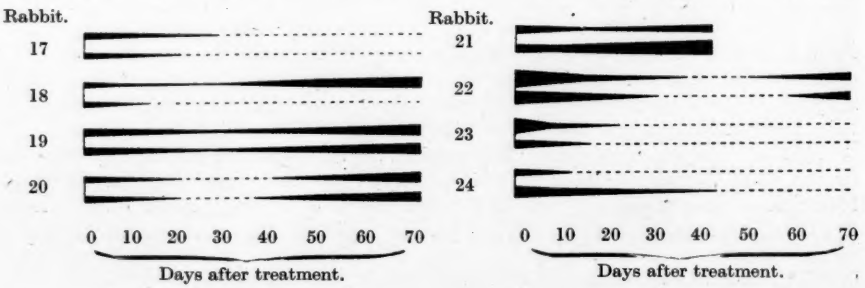
Expt. 3.—Neoarsphenamine, 2·5 mg. per kg., and penicillin, 80 days after inoculation.

Expt. 4.—Neoarsphenamine, 2·5 mg. per kg., and penicillin, 48 days after inoculation.



Expt. 5.—Neoarsphenamine, 5 mg. per kg., and penicillin, 27 days after inoculation.

Expt. 6.—Neoarsphenamine, 10 mg. per kg., and penicillin, 42 days after inoculation.



rabbits have any considerable effect on the results. The neoarsphenamine product used was novarsenobillon (May & Baker), and it was injected intravenously after being dissolved in normal saline to give the requisite dose per kg. in 1 c.c. The penicillin used all came from the same batch, and showed no loss of *in vitro* activity against the Oxford staphylococcus during the course of the experiments. It was dissolved in saline, like the neoarsphenamine, to give the requisite dose per kg. in 1 c.c., and was injected intramuscularly. As is shown in Fig. 1, a single dose of neoarsphenamine was given which varied from experiment to experiment, but was the same for all four rabbits in each experiment. In order to reduce the effect of the rapid excretion of penicillin the total dose of penicillin was given in three equally divided doses at intervals of 3 hours, the first dose being given at the same time as the single dose of neoarsphenamine. The total doses of pencillin given, as shown in Fig. 1, were the same in Expts. 3 to 6, but varied from rabbit to rabbit in each experiment, being 30,000, 15,000, 9000 and 4500 units per kg. from the first to the fourth rabbit respectively.

RESULTS.

The results of the main therapeutic experiments are shown in Fig. 1, where the effect of treatment is shown by the progress of the testicular lesions from the time of treatment onwards. As will be shown later, the other criteria of the effect of treatment, namely, the disappearance and reappearance of spirochaetes and the results of the serological tests, closely paralleled the course of the lesions, so that Fig. 1 in itself gives an adequate summary of the results.

Effect on Lesions.

Neoarsphenamine alone.

In Expt. 1, Fig. 1, it is shown that while 10 mg. of neoarsphenamine per kg. was sufficient to cause an almost complete initial disappearance of the lesions, there was a considerable proportion of relapses, which was higher than that previously experienced with this strain of *T. pallida*, probably because of enhanced virulence promoted by more rapid passage during the past two years. In Expt. 2, however, it will be seen that 20 mg. of neoarsphenamine per kg. was sufficient to cause all lesions to disappear with no relapses up to the end of the 70-day period of observation.

Combined neoarsphenamine and penicillin.

Expt. 3 and 4, in which the rabbits were treated in exactly the same way with 2·5 mg. of neoarsphenamine per kg. and graded doses of penicillin, provided an interesting contrast in that treatment was highly successful in Expt. 3 with no relapses, whereas Expt. 4 shows relapses in all but one rabbit. This striking difference can be attributed to the fact that the rabbits in Expt. 3 and Expt. 4 were treated at 80 and 48 days respectively after inoculation. The rabbits in Expt. 3 had probably reached a stage of the infection at which they had developed considerable immunity, and were thus much more able to cope with the infection than the rabbits in Expt. 4. This result further emphasizes the difficulties that have already been mentioned in assessing the value of treatment in rabbit syphilis (Selbie and Simon, 1944). In any case it may be concluded from the rapid

relapse of the lesions in three of the rabbits of Expt. 4 that the two drugs in the doses used were much less effective than the single dose of 10 mg. of neoarsphenamine used in Expt. 1.

In Expt. 5 the same graded doses of penicillin along with 5 mg. of neoarsphenamine per kg. gave results similar to those in Expt. 1 with 10 mg. of neoarsphenamine alone, while in Expt. 6 the effect of increasing the dose of neoarsphenamine to 10 mg. in combined therapy were perhaps slightly superior. It will also be noted in Expt. 6 that relapses occurred only in the two rabbits receiving the larger doses of penicillin. This lack of correlation between the dose of penicillin used and the result of treatment has also been observed in treatment with penicillin alone (Selbie and Simon, 1944).

Effect on Spirochaetes.

In all the experiments shown in Fig. 1 motile spirochaetes could not be detected in dark-ground examinations of aspirated samples of the local lesion after 24 hours from the commencement of treatment, except for one lesion in Rabbit 1, Expt. 1, and one in Rabbit 15, Expt. 4, in both of which, however, they could no longer be seen at 48 hours. Regarding the reappearance of spirochaetes in relapsing lesions, this could usually be detected at the weekly examination following that at which an increase in the size of the lesion was observed.

The rapid disappearance of spirochaetes in these experiments was to be expected, since each rabbit received at least 10 mg. of neoarsphenamine or 4500 units of penicillin per kg., either of which is the minimal effective dose sufficient to ensure an almost regular disappearance of motile spirochaetes within 24 hours (Selbie and Simon, 1944). It appeared necessary, however, to find how far the minimal effective doses of penicillin and neoarsphenamine could be reduced when the drugs were given simultaneously. For this purpose a number of syphilitic rabbits were given 2·5 mg. of neoarsphenamine and total doses of penicillin ranging from 300 to 2400 units per kg., and it was found that the absence of motile spirochaetes from the lesions 24 hours after the commencement of treatment occurred regularly only in those rabbits receiving 2400 units of penicillin. Thus, combined treatment with 2·5 mg. of neoarsphenamine and 2400 units of penicillin per kg. was as effective in causing a rapid disappearance of spirochaetes as 10 mg. of neoarsphenamine or 4500 units of penicillin per kg.

Effect on Sigma Reaction.

Positive reactions in the sigma test were not usually obtained until 3 to 4 weeks after inoculation, at a time when the local lesions were already well developed, having attained diameters of 0·5 to 1·0 cm. The sigma titre then increased with

TABLE I.—*Sigma Reaction Titres in Successfully Treated Rabbits.*

Number of days after treatment.	Sigma titres in rabbit No.:—												
	1.	5.	6.	7.	8.	9.	10.	11.	12.	14.	17.	23.	24.
0	20	60	42	20	5	8	11	8	11	30	7	17	20
6–8	14	25	3	5	1·5	0	1·4	1·7	4·2	17	3·0	13	10
21	3·0	2·0	1·5	1·5	0	0	2·0	0	0·9	0·6	0	5	2
35	1·0	0	0	0	0	0	1·0	0	0	1·0	0	..	..
49	0	0	0	0	0	0	0	1·5	0	1·5	0	..	..
70	0	0	0	0	0	0	0	0·6	0	0	0	..	..

TABLE II.—*Sigma Reaction Titres in Unsuccessfully Treated Rabbits.*

Number of days after treatment.	Sigma titres in rabbit No.:—										
	2.	3.	4.	13.	15.	16.	18.	19.	20.	21.	22.
0	8	60	5	50	30	17	9	12	17	17	20
6–8	3·0	57	3·3	30	7	17	4·0	10	14	5	8
21	2·0	5	0·6	0	1·0	1·0	1·0	2·0	2·0	5	0
35	4·2	10	0	10	..	..	3·0	2·0	2·0	..	..
49	0	2·5	12	..	..	..	17	3·0	40	..	..
70	..	5	..	..	..	..	7	15	15	..	..

the progress of the lesions, but, as a comparison of Fig. 1 and Tables I and II will show, there was no close parallelism between the sigma titres and the size of the lesions at the time of commencing treatment. The progress of the sigma titres after treatment is shown in Table I for the rabbits that were successfully treated, and in Table II for the rabbits in which relapses occurred. It will be noted that the sigma titres fell rapidly to the 21st day in both groups of rabbits, and that there was, at this time, no significant difference in titre between the two groups. At the 35th day differences became apparent in that the sigma reactions of the successfully treated rabbits were already negative or showed very low titres, whereas most of the unsuccessfully treated group showed no further drop or a rise in titre. At the 49th day the sigma titres of the successfully treated rabbits had all become negative or showed only small residual titres, while the tests in the unsuccessfully treated group had all shown an increase in titre. These tests, however, were of little prognostic value except as confirmatory observations, because increases in sigma titre were obtained only when measurable increases in the size of lesions or the reappearance of spirochaetes had been detected.

DISCUSSION.

Taking the results of these experiments as a whole, it may be concluded that the curative effect of a single dose of neoarsphenamine in rabbit syphilis has not been appreciably increased by the addition of a short course of penicillin. It has been shown, however, that the two drugs have a synergic action in the early stages of treatment, in that a disappearance of motile spirochaetes within 24 hours can be obtained with a quarter of the dose of neoarsphenamine and a half of the dose of penicillin required to produce this effect with either drug alone. This difference between the immediate and late effects of combined neoarsphenamine and penicillin therapy is similar to that found in previous experiments on the treatment of rabbit syphilis with penicillin alone, where rapid regression of the lesions was followed by recurrences, even with relatively high doses of penicillin (Selbie and Simon, 1944). Similar results have also been described by Ercoli and Lafferty (1944), who obtained rapid healing of primary lesions in rabbits with 4 to 6 intravenous doses of 33,000 to 47,000 units of penicillin per kg. given in 48 hours, but found that lymph nodes were still infective some time after treatment. It would therefore appear that our failure to show a definite synergic action of neoarsphenamine and penicillin in the late effects of treatment is due to the relatively temporary action of penicillin.

The sigma reactions of the sera of the treated rabbits were followed to see whether they might be of prognostic value, but, as has already been mentioned, they were only of confirmatory value—a finding that has been made by Probey (1932), using the Kahn test in the appraisal of the prophylactic treatment of

rabbit syphilis with neoarsphenamine. It is, however, of interest to note that 3 of the 11 unsuccessfully treated rabbits showed negative sigma reactions at 21 or 35 days after treatment, so that, even in these short-term experiments, a negative serological test did not differentiate between absence of infection and temporary quiescence. This parallels the well-recognized finding in human syphilis treated with arsenical drugs, that a reversal of a serological test to negative may be only temporary, and is no guarantee of cure.

It has been suggested in the previous report (Selbie and Simon, 1944) that the low curative action of penicillin in rabbit syphilis, as compared with the promising results obtained in the penicillin treatment of human syphilis, may be due to the rabbit lesion being relatively larger and denser in consistency, and therefore less readily penetrated by drugs from the blood stream than the human lesion. However, later reports of the results of penicillin treatment in human syphilis are tending to confirm the results of the rabbit experiments in that they show a certain proportion of relapses. In fact, it would appear from the recent report of Marshall (1945) that penicillin therapy has not yet proved capable of ensuring a proportion of cures comparable to that obtained with arsenic and bismuth therapy.

Increased dosage of penicillin may yet give more favourable results in human syphilis, but Marshall, pending further investigations, advocates the use of combined therapy, which has already proved highly successful in his hands. He has treated over 200 cases with 2·4 million units of penicillin given in 8 days, along with a " 20-day arsenoxide treatment " cut down to two-thirds of the usual dose, and the results, so far, appear better than with penicillin alone or with the arsenoxide treatment at the usual dose level. This method of treatment, or perhaps other forms of penicillin therapy, combined with arsenical drugs and bismuth, would therefore appear to be highly advantageous in that it makes use of the undoubtedly high antisyphilitic properties of penicillin and at the same time reduces the toxic effects of arsenotherapy.

The experiments described here may be of some value in deciding the relationship between the dose of penicillin and that of the arsenical drug to be used in combined treatment. It has been shown that increasing the dose of neoarsphenamine gave progressively improved results, whereas there was little correlation between the dose of penicillin and the effects of combined treatment. It would therefore appear advisable in clinical practice that, in combining arsenical and penicillin treatment, the dose of the arsenical drug should not be reduced to less than a half or perhaps two-thirds of the dose used in arsenotherapy alone.

SUMMARY.

Rabbits infected with *Treponema pallidum* were treated with a single dose of neoarsphenamine intravenously, either alone or combined with a short concurrent course of penicillin, given intramuscularly.

The dosage required to cause a regular disappearance of motile spirochaetes from dark-ground preparations within 24 hours was 2·5 mg. of neoarsphenamine and 2400 units of penicillin per kg. in combined treatment, and 10 mg. of neoarsphenamine or 4500 units of penicillin per kg. when either substance was given alone.

The curative action of neoarsphenamine was not appreciably increased by the addition of a short course of penicillin.

The application of these findings to the treatment of human syphilis is discussed.

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RE-TESTING OF A STRAIN (IF) OF MICE SPECIALLY SENSITIVE TO CARCINOGENIC AGENTS APPLIED TO THE SKIN.

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THE establishment of a strain of mice particularly susceptible to carcinogenic agents applied to the skin was described by Bonser (1938) in what will be later referred to as the first experiment. These mice (termed IF) were shown to develop benign tumours of the skin following the application of tar and certain chemical carcinogens at an earlier date than either stock mice or various inbred strains, but the tumours took longer to become malignant. Later generations of this strain were used by Bonser and Wainman (1940) in a second experiment, when it was found that both benign and malignant tumours appeared earlier than in the first experiment. It is to be noted, however, that the conditions under which the mice were kept were slightly different as regards diet and temperature.

The present third experiment was undertaken to determine whether there has been any appreciable change in the response of the skin of IF mice to carcinogens, during a period of six years, covering 14 generations of brother-sister mating. The weaker of the two pure carcinogens used previously, 3 : 4-benzpyrene, was chosen as being the more delicate index of any change. The main points under consideration are whether the mice still develop tumours early and, if not, whether the change lies in the time which elapses prior to the appearance of warts, or in the interval between their appearance and the occurrence of malignancy ; whether the wart which appears first is the first to become malignant ; and whether there are any significant sex differences.

In the interval between the first two experiments and the present one the breast tissue of this strain has been found to be particularly susceptible to carcinogenic agents (Bonser, 1940 ; Bonser and Orr, 1939 ; Orr, 1943).

METHOD.

Twenty-six IF mice, 13 of each sex, of the 29th–31st generations of inbreeding were used. The mice in Expt. 1 belong to the 12th and 13th generations of brother-sister mating, derived from an original female which developed early warts; those in Expt. 2 to the 15th–17th generations. Thus 14 generations of inbreeding took place in the six-year period between the second and third experiments. The mice were kept at a constant temperature of 65°F., and were fed on a liberal mixed diet comparable, as far as wartime standards allow, with that used in Expt. 1.

The non-epilated interscapular region was painted once weekly by means of a camel hair brush with an 0·3 per cent solution of 3 : 4-benzpyrene* in benzene. The area was examined at the same time for warts. Malignant change was estimated clinically and subsequently the tumours were examined histologically. Weekly diagrammatic records were kept of the number, position, regression or growth, ulceration or malignancy of the tumours.

RESULTS.

Comparison of response of present IF mice with earlier generations.

From Tables I and II it is seen that warts occur in present IF mice later and over a greater range of time than in either of the two previous experiments. This difference is strikingly illustrated by estimation of the time at which 50 per cent

TABLE II.—*Average Time of Appearance of Warts and Malignancy in Various Strains of Mice.*

	Average time of appearance of first wart.		Average interval between first wart and first malignancy in any wart.	Average time of occurrence of malignancy.	
	Number of mice.	Time in weeks.	Time in weeks.	Number of mice.	Time in weeks.
IF : first expt.	26	18·2	12·2	25	30·2
IF : second expt.	26	12·0	8·4	25	20·4
IF : present expt.	26	25·7	7·2	24	32·9
White label (Kreyberg)	16	19·4	8·2	16	27·6
Strong A	17	40·3	8·2	16	48·8
Black agouti	20	33·8	6·5	20	40·3

of survivors bore warts : IF Expt. 1 at 17½ weeks, IF Expt. II at 11½ weeks, and IF Expt. III at 25½ weeks. By contrast, the interval between wart appearance and the development of malignancy is reduced to 7·2 weeks, compared with 12·0 and 8·4 weeks respectively in the earlier experiments (Table II). Thus, on an average, there is only slight delay in development of malignant tumours in later generation IF mice compared with generations 12 and 13, but marked delay compared with generations 15–17 (Table III).

Comparison of response of present IF mice with other strains used in Expt. 1.

A marked difference in time of appearance of warts and malignant tumours was noted between IF mice of the two previous experiments compared with a

* Supplied by Messrs. L. Light & Co., Wraysbury, Bucks, as in the previous experiments.

TABLE I.—*Appearance of Warts in Various Strains.*

Weeks.	IF. First expt.		IF. Present expt.		White label (Kreyberg). First expt.		Strong A* First expt.		Black agouti. First expt.		IF. Second expt.	
	Number of mice developing warts.	Per cent.	Number of mice developing warts.	Per cent.	Number of mice developing warts.	Per cent.	Number of mice developing warts.	Per cent.	Number of mice developing warts.	Per cent.	Number of mice developing warts.	Per cent.
0–12	1	3·8	—	—	1	6·3	—	—	—	—	17	65·4
13–16	6	23·1	2	7·6	3	18·9	—	—	—	—	9	34·6
17–20	15	57·7	2	7·6	4	25·2	—	—	1	5·0	—	—
21–24	3	11·6	5	19·2	6	37·8	—	—	1	5·0	—	—
25–28	1	3·8	11	43·3	2	12·6	1	5·9	—	—	—	—
29–32	—	—	4	15·2	—	—	1	5·9	6	30·0	—	—
33–36	—	—	1	3·8	—	—	2	11·8	3	15·0	—	—
37–40	—	—	—	—	—	—	4	23·6	8	40·0	—	—
41–44	—	—	1	3·8	—	—	3	17·7	1	5·0	—	—
45–48	—	—	—	—	—	—	5	29·5	—	—	—	—
49–52	—	—	—	—	—	—	1	5·9	—	—	—	—
Total	26	—	26	—	16	—	17	—	20	—	26	—

* By an error this strain was previously termed Bagg albino (Bonser, 1939–40; Bonser, 1940).

TABLE III.—*Occurrence of Malignancy in Various Strains.*

Weeks.	IF. First expt.		IF. Second expt.		IF. Present expt.		White label (Kreyberg). First expt.		Strong A. First expt.		Black agouti. First expt.	
	Number of mice developing malignant tumours.	Per cent.	Number of mice developing malignant tumours.	Per cent.	Number of mice developing malignant tumours.	Per cent.	Number of mice developing malignant tumours.	Per cent.	Number of mice developing malignant tumours.	Per cent.	Number of mice developing malignant tumours.	Per cent.
0–12	—	—	—	—	—	—	—	—	—	—	—	—
13–16	—	—	5	20·0	—	—	1	6·3	—	—	—	—
17–20	—	—	8	32·0	—	—	—	—	—	—	—	—
21–24	2	8·0	11	44·0	2	8·4	2	12·6	—	—	—	—
25–28	10	40·0	1	4·0	5	21·0	6	37·8	—	—	1	5·3
29–32	4	16·0	—	—	2	8·4	5	31·5	1	6·3	1	5·3
33–36	5	20·0	—	—	9	37·8	2	12·6	—	—	4	21·2
37–40	4	16·0	—	—	4	16·8	—	—	—	—	1	5·3
41–44	—	—	—	—	1	4·2	—	—	2	12·6	8	42·4
45–48	—	—	—	—	—	—	—	—	6	37·8	3	15·9
49–52	—	—	—	—	1	4·2	—	—	2	12·6	1	5·3
53–56	—	—	—	—	—	—	—	—	3	18·9	—	—
57–60	—	—	—	—	—	—	—	—	1	6·3	—	—
61–64	—	—	—	—	—	—	—	—	1	6·3	—	—
Total	25	—	25	—	24	—	16	—	16	—	19	—

high breast cancer strain (Strong A) and a low breast cancer strain CBA). This difference, though of a lesser order, is maintained in the present experiment (Tables I, II and III). But when compared with Kreyberg's "white label" strain it is seen that a difference which was equivocal in previous experiments now becomes such that both warts and malignant tumours occur later in IF than in "white label" mice.

Relation between the first wart and the subsequent development of malignancy.

In the previous experiments the interval between wart appearance and the development of malignancy was based on the time between the appearance of the first wart and the inception of malignancy in any wart. This has been estimated in the present experiment, but in addition the interval between the appearance of each individual wart and the onset of malignancy in it has been calculated. In examining each mouse it was not unusual to find that even where two warts had appeared at separate dates and in separate sites, the malignant change would cause the formation of a mass in which both the warts were incorporated. In these cases the interval was calculated between the date of appearance of the first or second wart and the development of the malignant mass. It is seen (Table IV)

TABLE IV.—*Average Intervals Between Wart Appearance and Development of Malignancy in IF Mice of Present Experiment.*

Interval between—	No. of mice.	Average interval in weeks.
1st wart and 1st malignancy in any wart . . .	24	7·2
1st wart and 1st malignancy in that wart . . .	24	7·4
2nd wart and 1st malignancy in that wart . . .	13	7·6
3rd wart and 1st malignancy in that wart . . .	3	7·0

that there is no significant difference in the time interval of the malignant change in first, second or third warts. It was, however, more difficult to determine whether the first wart to appear was the first to become malignant. Among cases which were indeterminate were those in which there was only one wart (three mice); or where two or more warts appeared and became malignant simultaneously and there were no later tumours for comparison (three mice); or those in which only one of several warts appearing simultaneously showed malignant change (two mice); and finally those mice (four) in which malignancy supervened simultaneously in warts which had arisen at different times. Two mice, each bearing three warts, failed to develop malignancy before death at 43 weeks.

Of the remaining twelve clear-cut cases, nine first warts, two second and one third wart developed malignancy first. Thus it would seem that the original method of calculation of the time interval is reasonably accurate.

Accuracy of the clinical estimation of malignancy.

Of 41 warts examined 35 were estimated clinically to be malignant and this was confirmed by histological examination. One wart of large size was clinically malignant but microscopically simple, and the other five, though regarded as simple, proved to be malignant histologically.

Influence of sex.

In none of the experiments using IF mice (Table V) does there appear to be any significant difference between the sexes in time of appearance of warts or

TABLE V.—*Influence of Sex on the Time of Appearance of Warts and Malignancy in IF Mice.*

		Average time of appearance of first wart.		Average interval between appearance of first wart and malignancy in any wart.	Average time of occurrence of malignancy.	
		No. of mice.	Time in weeks.	Time in weeks.	No. of mice.	Time in weeks.
First expt., 12th and 13th generations	Female	12	18·4	12·3	11	30·7
	Male	14	17·9	11·8	14	29·7
Second expt., 15th–17th generations	Female	11	11·5	8·6	11	20·1
	Male	15	12·5	8·2	14	20·7
Present expt., 29th–31st generations	Female	13	24·7	7·2	11	32·1
	Male	13	26·7	7·1	13	33·8

onset of malignancy. The occasional failure to develop a malignant tumour occurred in three female and one male mouse.

DISCUSSION.

The main object of the experiment was to find out whether the lapse of a period of six years, during which time IF mice had not been tested, had had an effect upon the response of the skin to carcinogenic agents. The unexpected result has been obtained that the average time of appearance of warts is delayed, whereas the interval between wart formation and the subsequent development of malignancy is reduced. A comparison of early and late generations of IF mice is rendered more difficult by the fact that the two groups from the early generations gave a different response, the conditions under which these mice were kept being slightly different as regards diet and temperature (Bonser and Wainman, 1940). The tendency of the later group in the earlier generations was towards an earlier response, whereas the tendency now is towards a later response.

Despite the delay in appearance of warts in the IF strain at the present time, both warts and malignant tumours still arise considerably earlier than in the Strong A and black agouti strains when tested at the time of the first experiment (Tables I and III).

The occurrence of the change can hardly be attributed to differences in husbandry, in that the conditions of the present experiment conformed as nearly as wartime food would allow to those of the first experiment. A more possible explanation would appear to be the fact that, during the war for a number of reasons, the stock of IF mice has been kept very low so that a considerable, though random, selection has been exercised. To restore the characteristic of early wart appearance it would seem to be necessary to institute a deliberate selection of litters from animals which themselves develop early warts, which was the method by which the strain was first started.

The attempt to discover whether the wart which appears first is the first to become malignant was handicapped by the fact that half of the 24 mice available could not be included in the analysis for the reasons stated above. But in three-quarters of the 12 suitable cases the first wart to appear was the first to become malignant. Additional evidence was obtained that there was no appreciable difference in the interval between the time of appearance of first, second and third warts and the onset of malignancy in those warts respectively (Table IV).

As has been previously shown (Bonser, 1938), the clinical estimation of the malignant change appears to be sufficiently accurate for the purpose of experiments of this kind.

SUMMARY.

Mice of a strain (termed IF) particularly susceptible to carcinogenic agents applied to the skin have been re-tested after the lapse of six years, covering 14 generations of inbreeding, during which time considerable, though random, selection has been exercised in maintaining the strain. The characteristics of early wart formation and delayed development of malignancy have been considerably modified.

Evidence has been obtained that the first wart to appear is usually the first to become malignant, and that there is no difference in the response of the two sexes of this strain to skin carcinogens.

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THE INCIDENCE OF RH TYPES IN THE BRITISH POPULATION.

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LANDSTEINER and Wiener (1940) divided people into two groups, depending upon the ability of their erythrocytes to be agglutinated by the sera of rabbits immunized with Rhesus monkey blood. The following year they showed that the antigen demonstrated by these sera was inherited in a simple Mendelian manner.

Since then several different types of human immune Rh sera have been found as a result of isoimmunization in pregnancy or due to transfusion.

The prolific writings about Rh subgroups with numerous changes in termi-

nology have made the subject not only complicated, but also have made difficult the comparison of findings between different workers in various populations.

Briefly, the present position is that there are several different Rh antigens, each of which is capable of inducing immunity in any human lacking that antigen. Six Rh agglutinins have been found, and with their aid it is possible to detect the presence of the corresponding antigens in red cells or tissue cells by agglutination or absorption experiments.

The only satisfactory explanation of the problem is the ingenious scheme of Fisher (Race, 1944), who used the terms $\Gamma\Delta H\gamma\delta\eta$ for antibodies and CDEcde for the corresponding antigens. (At that time only the first four of these antibodies had been found.) Fisher's terminology has led to some confusion because of the dual system of lettering, and many people have wrongly assumed that the use of large and small lettering denotes dominant and recessive characters.

For simplicity I labelled Rh antibodies numerically (1 to 6) (Murray, 1944*a*), and suggested that the antigens they detected in cells should have the same number.

So far as we know at present, individuals can have in their cells three, four, five or six Rh antigens, and Fisher has deduced from the findings of Race and others that each individual antigen is inherited by a gene, and that genes are transmitted in groups of three on a chromosome. It is clear then that everyone has in their cells a pair of chromosomes, each bearing three positions or loci for Rh genes.

When the numerical notation was proposed the same number was used for the antibody, the antigen and the gene conferring its presence, and at that time I supposed that each chromosome carried the three genes with the loci arranged in the order shown in Fig. 1.

Loci	2 Chromosomes.	
Top	1 or 4	1 or 4
Middle	2 or 5	2 or 5
Bottom	3 or 6	3 or 6

FIG. 1.—The order of loci for Rh genes on the chromosomes.

According to my interpretation of Fisher's hypothesis, genes 1 and 4 are alternatives for the top locus, can only occupy that position, and are called alleles, genes 2 and 5 are alleles for the middle locus, and genes 3 and 6 are alleles for the bottom locus.

It is important to note that each pair of alleles are equally dominant and there are no recessive genes.

1	1	1	1	4	4	4	4
5	2	2	5	5	2	2	5
6	6	3	3	6	6	3	3
	*						

* This triad of genes has not yet been found segregated.

FIG. 2.—The eight possible triads composed out of six genes.

The reactions of the antigens conveyed by the eight triads of genes with six antibodies are shown in Table I, where the numerical terminology is compared with Fisher's notation and previously used terms.

(In this paper h is omitted from Rh symbols for simplicity in tables and figures.)

TABLE I.

Genetic antigens. Common name.	Fisher.	Murray.	Antibodies. Fisher / Murray	Γ 1.	H 2.	Δ 3.	γ 4.	η 5.	δ 6.
R′	Cde	156		+	−	−	−	+	+
R_y	CdE	126		+	+	−	−	−	+
R_z	CDE	123		+	+	+	−	−	−
R_1	CDe	153		+	−	+	−	+	−
R″	cdE	426		−	+	−	+	−	+
R_2	cDE	423		−	+	+	+	−	−
R_o	cDe	453		−	−	±	+	+	−
r	cde	456		−	−	−	+	+	+

The present investigation was undertaken to discover the incidence of Rh types, and the bloods of 1038 unselected people from the London area have been examined. With the assistance of the numerical notation the results will be presented and the origin of rare types discussed.

METHODS.

The specific anti-Rh sera used were human immune sera of the four types 1, 2, 3 and 4, the reactions of which are shown in Table I. Two or more examples of each of these were used, but for statistical analysis the reactions of only one No. 1 serum were used, because this serum called " Brown " was considered to be the only pure No. 1 which had been used throughout. There were some occasions when potent No. 2 sera were scarce and only one serum of this type could be used.

The method of testing was similar to that previously described (Murray, 1944*b*). Briefly, the method consists in incubation for three hours at 37° C. of a mixture of equal quantities (0·01 ml.) of antiserum and 2 per cent suspension of red cells in saline. The tests were made in tiny test-tubes measuring 2 in. × 0·2 in., and the deposits were examined for agglutination under the 2/3 in. power of the microscope. Multiple grooved slides were used for the examination of the cell deposits as previously described.

RESULTS.

The statistical results are presented in Table II, Table III and Table IV.

TABLE II.—*Reactions of Red Cells from* 1038 *Persons with Rh Antibodies* 1, 2, 3 *and* 4.

Reactions with antibodies. 1.	2.	3.	4.	Phenotype.	Total.	Percentage.	Most common type in each group.
+	−	+	+	R_{134}	354	34·10	153/456 R_1r
+	−	+	−	R_{13}	215	20·71	153/153 R_1R_1
−	−	−	+	R_4	159	15·31	456/456 rr
−	+	+	+	R_{234}	153	14·74	423/456 R_2r
+	+	+	+	R_{1234}	119	11·46	153/423 R_1R_2
−	−	+	+	R_{34}	24	2·31	453/456 R_or
+	−	−	+	R_{14}	6	0·58	156/456 R′r
−	+	−	+	R_{24}	7	0·67	426/456 R″r
*+	+	+	−	R_{123}	1	0·096	153/123 R_1R_z

* 123/456 (R_{zr}), which must be nearly as common as 153/123 (R_1R_z), is indistinguishable from 153/423 (R_1R_2) without the assistance of serum 6 (Fisher's δ).

The percentage occurrence of Rh antigens 1, 2, 3 and 4 in the bloods of this series are shown in Table III compared with the figures obtained by Race (Fisher and Race, 1946).

TABLE III.—*The Frequency of Rh Antigens in the British Populace.*

Antigen.	Per cent. detected.		Gene frequency from author's figures.
	Murray (1038 bloods).	Race (927 bloods).	
1	66·95	69·26	0·6695
2	26·97	27·18	0·2697
3	83·42	83·28	0·8342
4	79·17	80·15	0·7917

Examination of Table III shows how closely the findings of Race and the author fit. Only in the case of antigen 1 do the figures vary much, and this may be due to the fact that only the reactions of the No. 1 serum "Brown" were taken for the author's analysis, as already stated. Some of the other available sera containing the No. 1 antibody also contained varying amounts of another antibody, which has been described as anti-C^w (Callender, Race and Paykoç, 1945).

The frequencies of the gene triads calculated from Table II are shown in Table IV. For example the frequency of 456 is found thus:

The percentage occurrence of the homozygote 456/456 = 15·31
Therefore the frequency of the homozygote 456/456 = 0·1531
And the frequency of the gene triad 456 = $\sqrt{0·1531}$
= 0·3931

TABLE IV.—*Frequencies of Gene Triads and Percentages of Homozygotes Calculated from Percentages of Phenotypes.*

Gene Triad.	Frequency.	Homozygote.	Percentage.
153 . R_1	= 0·4551	153/153	= 20·71
456 . r	= 0·3931	456/456	= 15·31
423 . R_2	= 0·1270	423/423	= 1·60
453 . R^y	= 0·0231	453/453	= 0·50
156 . R'	= 0·0074	156/156	= 0·05
426 . R''	= 0·0078	426/426	= 0·06
123 . R_z	= 0·0011	123/123	= very rare.

Since the homozygote 423/423 was not capable of being identified separately in this series the frequency of the gene triad 423 was calculated from the percentage found for the serologically distinguishable types, or phenotypes, R_{1234} and R_{423}, and since each of these phenotypes covers a number of individual types the figure 0·1270 given for the frequency of 423 is only approximate.

Examination of Tables II and III shows that the commonest Rhesus types can be expressed as in Fig. 3 in order of frequency.

	R_1. r.	R_1. R_1. r. r.	R_1. R_2. r.	R_2. r.
Position of genes on chromosome	1 4 / 5 5 / 3 6	1 1 / 5 5 / 3 3	4 4 / 5 5 / 6 6	1 4 / 5 2 / 3 3	4 4 / 2 5 / 3 6
Approximate percentage of population	35	20	15	12	10

FIG. 3.—The five commonest Rh types in order of frequency.

It will be appreciated that any type containing gene 3 is included in the group which has been called "Rh positive," since serum 3 reacts in the same way as the original standard anti Rh of Landsteiner and Wiener.

DISCUSSION.

It has been suggested (Wiener, 1942) that the distribution of Rhesus types in different parts of the world could be produced by the intermarriage of races consisting either chiefly of Rhesus positive or Rhesus negative people.

Now Fisher and Race (1946) have proposed that the rare types 156, 123 and 426 can be produced by crossover of genes as shown in Fig. 4.

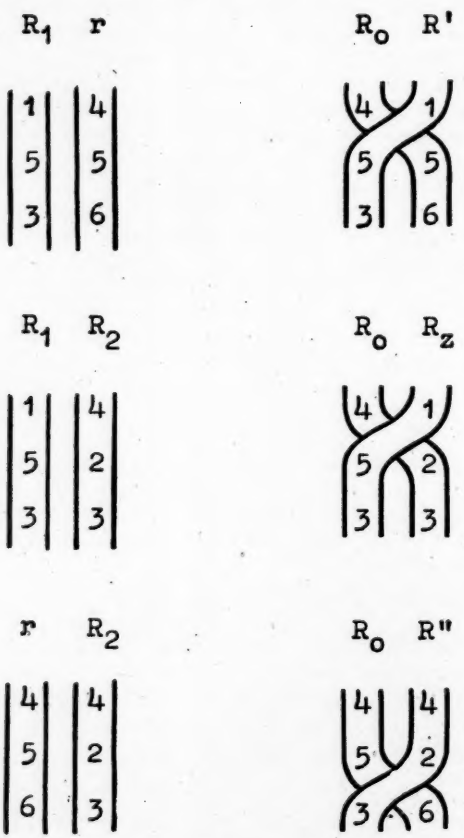

FIG. 4.—The method of production of uncommon gene triads from common ones by crossover.

They say that the gene triad 453 is nearly as common as 156 + 123 + 426, and I have found that the incidence of 453 is greater than the sum of the incidence of these three. It is difficult to see why Fisher and Race suggest that the c locus (4 in my terminology) should lie between e (5) and d (6), and I would suggest that the order of loci is as I have placed them in Fig. 1, because if the 1–4 locus (Cc) lies in the centre of the three, as Fisher and Race suggest, the production of

156 and 123 by crossover would involve double crossover in each case as can be seen in Fig. 5, which means that 426 should be very much more common than 156, and this is not in accordance with my findings. In this series 426 is found as often as 156 and much more often than 123.

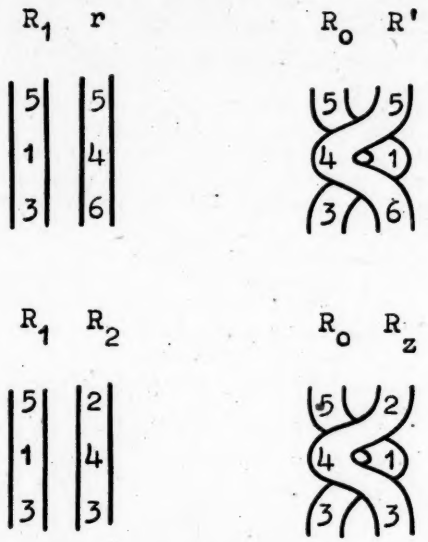

FIG. 5.—The figure shows double crossovers entailed by the order of genes suggested by Fisher and Race.

Matings involving 153 and 456 and so capable of producing 156 are more than twice as common as matings involving 423 and 456, which may produce 426 by single crossover in each case. But Race finds 426 occurring twice as often as 156, while I find them equally often instead of half as often, as would be expected if the genes were equally spaced on the chromosome. It appears that the chance of a crossover occurring to produce 426 is at least twice that of the chance of crossover producing 156, and this could be explained by a gap in the chromosome strip between the 2–5 (E–e) locus and the 3–6 (D–d) locus, thus giving greater chances for a crossover occurring at the 3–6 (D–d) locus (Fig. 6).

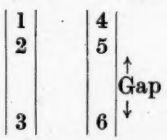

FIG. 6.—Two chromosomes are shown with the suggested gap between the middle and bottom loci for genes.

The superiority of frequency of 156 over 123 is partly explained by the matings involving 153 and 456 being more frequent than matings involving 153 and 423, as will be appreciated from their figures in Table IV.

It is plain from Tables II and IV and from Fisher's and Race's table that the frequency of 426 is nowhere near so great in relation to 156 and 123 as it would be if these two were produced by double crossover. It seems that the frequency of the gene 453 is greater than 156 + 426 + 123, and I think it reasonable to suppose that every time each of these three gene triads is produced 453 is also produced by the crossover.

Fig. 7 shows the commonest groups of three genes.

	R_1.	r.	R_2.
	1	4	4
	5	5	2
	3	6	3
Frequency	0·4551	0·3913	0·127

FIG. 7.

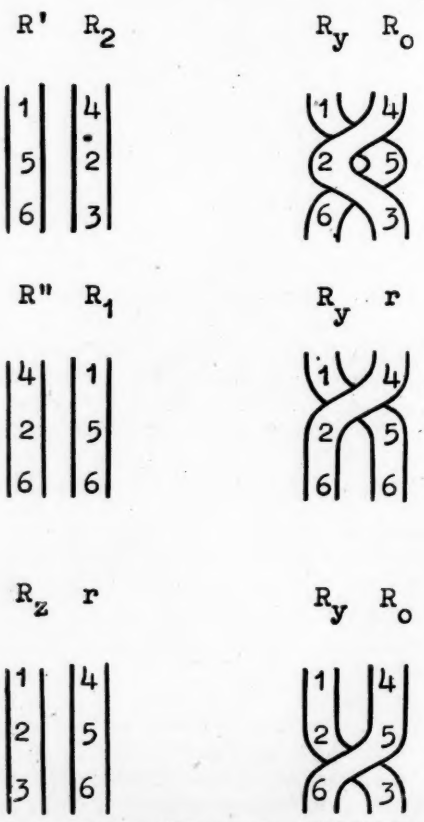

FIG. 8.—The three methods of production of the rare gene triad 126 from uncommon triads.

It can be surmised that the distribution, so far as it is known in the world of the 6 Rh antigens, could be arrived at by intermarriage of "pure types," i.e. homozygotes of 153, 456 and 423, with subsequent occasional crossover producing four other and rarer combinations of genes 453, 156, 426 and 123; 126 being the rarest combination, and so far undetected for lack of serum 6, is produced as a result of crossover involving the three combinations of genes 156, 426 and 123, which have already themselves been produced by crossover (Fig. 8). This, I agree with Fisher and Race, explains the extreme rarity of 126. As one of the three methods involves double crossover in the second stage, it can be disregarded as a method of production except in extremely rare circumstances.

Since I have suggested that the 3–6 (D–d) locus is separated by a gap, the most likely method of production of 126 is in the 123/456 combination.

This theory of three original human types can be made to include the third allele 7 at the 1–4 locus (Cc), which has recently been described as C^w by Race and his colleagues (1945), and it could also include the possible alleles 8 and 9, which may occur at the 2–5 and 3–6 loci respectively. In order to fit in the allele 7 to this theory mutation must have occurred at the 1–4 locus.

If genes 8 and 9 have been produced by mutation and are as yet unrecognized, due to their rarity, or if they occur in the future, then the reactions of their antigens and corresponding antisera can be shown as in Table V, which is an extension of the original symmetrical scheme.

TABLE V.—*The Reactions of Antisera* 1 *to* 9 *with* 27 *Groups of Three Antigens.*

		Antisera.								
		1.	2.	3.	4.	5.	6.	7.	8.	9.
		C.	E.	D.	c.	e.	d.	C^w.		
156	r	+	−	−	−	+	+	−	−	−
126	R_y	+	+	−	−	−	+	−	−	−
123	R_z	+	+	+	−	−	−	−	−	−
153	R_1	+	−	+	−	+	−	−	−	−
426	R″	−	+	−	+	−	+	−	−	−
423	R_2	−	+	+	+	−	−	−	−	−
453	R_o	−	−	+	+	+	−	−	−	−
456	r′	−	−	−	+	+	+	−	−	−
726		−	+	−	−	−	+	+	−	−
723		−	+	+	−	−	−	+	−	−
429		−	+	−	+	−	−	−	−	+
183		+	−	+	−	−	−	−	+	−
129		+	+	−	−	−	−	−	−	+
159		+	−	−	−	+	−	−	−	+
189		+	−	−	−	−	−	−	+	+
729		−	+	−	−	−	−	+	−	+
186		+	−	−	−	−	+	−	+	−
783		−	−	+	−	−	−	+	+	−
483		−	−	+	+	−	−	−	+	−
753		−	−	+	−	+	−	+	−	−
756		−	−	−	−	+	+	+	−	−
786		−	−	−	−	−	+	+	+	−
486		−	−	−	+	−	+	−	+	−
759		−	−	−	−	+	−	+	−	+
789		−	−	−	−	−	−	+	+	+
489		−	−	−	+	−	−	−	+	+
459		−	−	−	+	+	−	−	−	+

It is unlikely that the human race started as a single pure stock and that mutation has occurred at each of the three loci producing the alleles 1, 4 or 7 for

the top locus, 2 or 5 for the middle locus and 3 or 6 for the bottom locus because, if this were so, it would be expected that the incidence of the genes would be very much more varied than is found.

Only in the case of gene 7 can mutation be considered, as gene 7 appears to occur in only 1·5 per cent of people, according to Callender, Race and Paykoç (1945), whereas its alleles 1 and 4 occur in 67 per cent and 80 per cent of people in this country.

Attractive though it would be to find that the origin of Rhesus types corresponds with white, yellow and black races, there is no evidence that such is the case, although there is a great difference in frequency of the individual Rh antigens in whites, negroes and yellow people.

SUMMARY.

1. 1038 bloods were examined for Rh antigens, using four different types of Rh antibodies.

2. The results are interpreted with the author's numerical notation, the advantages of which are emphasized.

3. A statistical analysis of the results is given.

4. Reasons are given for suggesting the order of Rh genes on a chromosome, and the spacing of loci to be unequal.

5. The proposal of Fisher and Race that rarer types could have been produced from the common Rh types by crossover of genes is demonstrated, and the order of loci suggested by them is criticized.

6. The theory of production of rare types is shown to be consistent with the occurrence of further and rarer genes produced by mutation; for example, the mutant C^w described by Callender and others (1945).

7. The possible origin of man from three pure stocks is discussed.

8. Predicted reactions of further possible antigens caused by mutant genes at the middle and bottom loci on the chromosomes are given.

I would like to thank Prof. J. McIntosh for his stimulating interest. My grateful thanks are also due to the late Dr. G. L. Taylor and Dr. R. Race for their invaluable help, and I am grateful to Prof. D. F. Cappell, and Misses K. Boorman and B. Dodd and Dr. A. S. Wiener for the exchange of antisera.

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THE RELEASE OF ANTIBODY BY SENSITIZED ANTIGENS.

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SEVERAL workers have shown that some antibody can be released from an antigen-antibody complex after union. Huntoon and Etris (1921) found that antibody could be released from sensitized antigen in 10 per cent saccharose or in distilled water. Uchida (1927) showed that small amounts of *B. typhosus* agglutinin and amboceptor were dissociated from sensitized antigen by solutions of glucose, saccharose, or physiological saline solution. The optimum temperature for release was 55–56° C. The final solution contained a doubtful trace of protein and 4·9 to 8·4 mg. per cent of nitrogen. He found that the released antibody was not dialyzable and was not affected by trypsin digestion. It may be remarked here that if the nitrogen found represented globulin, an appreciable amount must have been present, much more than could be described as a doubtful trace.

Further work was done by Nelson (1928), who states that heating sensitized bacteria to 55° C. in 5 per cent saline solution resulted in the removal of considerable flagellar agglutinin. Whilst it was free in the sense that it could combine with specific antigen, Nelson thought it might still retain some cellular fraction from its previous union. He presents evidence which he considers to show that agglutinin release is not solely dependent upon the disintegration of flagella. He found that little or no somatic agglutinin was released.

The published descriptions of released antibody all indicate that the concentrations obtained were very weak. It has been found possible to prepare samples of considerable titre, and it is not necessary to use sugar solutions to secure release. The amount of antibody taken up when an excess is presented to the antigen varies inversely with the temperature, and appreciable amounts can be freed by heat alone if absorption is carried out at 0–10° C., the sensitized mass washed at that temperature, and finally heated at 56° C. Agglutination proceeds slowly at temperatures near 0° C., and the purpose of keeping the mass cold and of rapidity in manipulation is to bring it to a state fit for agglutination at 56° C. with the minimum loss of released antibody. The reaction can be further delayed by using minimal concentrations of electrolyte. It is therefore advantageous to use for washing 0·02 per cent NaCl instead of the usual 0·88 per cent.

The union between *B. typhosus* and its antibody at low temperature is almost instantaneous; once they have been mixed there is no process sufficiently rapid to separate them before union. The only practicable course is to delay the end.

The rate of union varies with different organisms. *B. paratyphosus* A and B require 5 minutes for saturation, and *B. dysentery shiga* about 20 minutes.

Preparation of Released Antibody.

Eight c.c. of a 20,000 million per c.c. suspension of *B. typhosus* is placed in a centrifuge tube and cooled in a beaker of melting ice. Then 1 c.c. of high titre undiluted antiserum is added and the tube centrifugalized in an angle centrifuge at once. In 4 minutes the sensitized mass will be separated and the supernatant serum is poured off. The mass is then washed three times with 0·02 per cent saline similarly chilled, and finally resuspended in 5 c.c. of normal saline. It is placed in the 56° C. bath for 15 minutes and shaken once or twice during that time. Finally it is separated in the centrifuge, and the supernatant, containing the released antibody, collected.

To secure the release of antibody it is a fundamental requirement that the antigen shall be able to take up the maximum amount of antibody; consequently there should always be a substantial amount left in the supernatant after the first separation. If all the antibody has been absorbed agglutination may proceed normally, but no antibody will be released later.

Three washings are usually enough, but the precise number is determined by the titre of the original serum. After each supernatant is decanted the fluid remaining in the bottom of the tube is 0·25 to 0·35 c.c., and since the tube has a capacity of 10 c.c. it follows that the antiserum remaining in free solution is diluted about 30 times when the next volume of saline is added. It may be taken as a general working rule that the total serial dilutions should be at least 20 times the known end-point of the antiserum.

The titre to be expected from 5 c.c. of released antibody (RA) prepared as above is about 1 in 16. Some is lost in the washings mixed with the residue of the unabsorbed antiserum.

Released antibody is perfectly transparent: any turbidity is due to accidental inclusion of some of the agglutinated mass, and should be removed by further centrifugalization. Its stability is not as great as that of ordinary antiserum and it should be stored frozen solid. On the first occasion of thawing for use it is usually faintly turbid and the deposit should be removed in the centrifuge. The titre is not affected, and no turbidity forms after any subsequent freezing and thawing.

Method of Preparing Concentrated Solutions of Released Antibody.

Two procedures are available and they depend upon the fact that, unlike fresh antiserum, RA once shed will not reunite with the sensitized mass at low temperature.

The summation method is described first. The first stage of this method is the same as that already described. The RA obtained is set aside. The agglutinated mass is next treated again with antiserum and the process of washing and release by heating repeated, but instead of using a volume of saline into which to discharge the RA, the original RA is used. In the bath a second dose of RA is discharged into the solution and its titre thereby doubled. Three or four further stages are carried through in the same way, and it will be found a simple

matter to obtain solutions with a titre of two or three hundred. The highest titre obtained in this work was 1 in 640.

Prolonged use of the method is wasteful because incessant cooling and heating of RA and repeated contact with particulate matter destroys it. In the later extracts the loss equals the gain and finally may actually exceed it.

The high concentration method is preferable. A massive concentration of sensitized antigen should be used. The saline for extracting the RA is restricted to about 2·0 c.c., and further extractions are each made with a similar volume of fresh saline. When the collected volume amounts to 7 or 8 c.c. it is permissible to use the solution for one further extraction as in the summation method. The sensitized mass should not be used more than about eight times.

The manner in which RA is discharged is shown by the following experiment: *B. typhosus* suspension and antiserum were mixed to form a saturated agglutinated system, a low temperature was maintained throughout the washings, and the washings were saved. After the final washing the RA was collected at 58° C. RA differs from fresh antiserum by reason of its inability to pass a porcelain filter, so the titre of each fraction was taken before and after filtration in order to distinguish between the two types of antibody.

Titre of Fractions.

	Before filtration.	After filtration.
First supernatant (surplus serum)	640	640
First washing	32	32
Second ,,	8	2
Third ,,	16	0
Fourth ,,	32	0
Released at 58° C.	48	0

The agglutination proceeds throughout the manipulations of the mass, and the RA is shed continuously and at an increasing rate as the reaction advances. At first the antibody is of the seral type as shown by its passage through the filter, and it represents the remnant in free solution which has not yet been washed away.

If the units of antibody released at 58° C. are compared with the units of the original seral antibody absorbed it will be found that the RA is 4 to 10 per cent of the latter: nearly twice as much RA is lost in the washings. It follows that when a suspension of *B. typhosus* is fully saturated at low temperature, it releases about a quarter of its flagellar antibody by the time agglutination is complete at 58° C. The generalization, although inexact, is derived from observation of a large number of preparations.

The incessant washing in a multistage extraction causes minute losses of material, which in sum amount to an appreciable proportion of the original mass. This loss can be diminished by using 0·02 per cent saline to which 1 part in 100 of a 1 per cent solution of saponin has been added. Investigation confirmed Takenomata's finding (1924) that saponin has no influence on agglutination. The stock 1 per cent solution should be preserved by adding 0·2 per cent of formaldehyde. In 24 hours a thick turbidity forms which can be removed by the centrifuge. The solution will then remain permanently clear.

Examination of Released Antibody.

The titres to be expected from RA have been indicated, and its specificity is in every way equal to that of the antiserum from which it is derived.

Released antibody can be distinguished from normal antiserum by several characters. It cannot be filtered through fairly fine grain porcelain, and the observation supports Uchida's finding that it will not dialyse. A Jenkins filter was used, as it permits of the handling of small volumes and is quickly cleaned and reassembled.

The fluid passed by the filter has no agglutinating power, and if any small titre remains it can be removed by a second passage. Single passage has been found enough to establish the essential distinction in any sample. Some typical results are :

	Flagellar titre.	
	Before filtration.	After filtration.
RA of *B. typhosus* . . .	160	2
B. paratyphosus A . .	160	0
,, *B* . .	80	0
,, *C* . .	32	0
B. enteriditis gaertner .	64	2

The attraction of the agglutinating factor for the surface of the filter is paralleled by its adsorption to other particulate matter, including kaolin and bacteria with which it has no known antigenic affinity. This matter is mentioned only in order to make clear that the behaviour of RA to indifferent particulate matter is not analogous to its union with homologous antigen.

The routine technique for demonstrating non-specific adsorption is as follows : Two c.c. of a heterologous suspension, having an apparent strength about 8 times that of a normal antigenic suspension, is mixed with 6 c.c. of RA having a titre of not less than 1 in 16. The staphylococcus is a convenient organism. The mixture is left on the bench for 40 minutes, shaking gently at intervals. It has been found that 15 minutes is the minimum which offers any hope of adsorption. The suspension is next washed 2 or 3 times to remove any RA in free solution, and the final deposit resuspended in 2 c.c. of saline. Spontaneous agglutination of this sensitized suspension has never been seen.

The suspension is then used as if it were a solution of antiserum, and titrated against the antigen homologous with the selected RA. Agglutination is specific, and is always found in 1 in 2, usually in 1 in 4, and rarely in 1 in 8.

Three points differentiate this type of reaction from true specific absorption :

1. It is necessary to offer an area of particulate matter at least 8 times that of the homologous antigen.
2. The time taken for adsorption is far longer than that for specific union.
3. The amount of RA taken up and subsequently released to react with its homologous antigen is small judging by the low titres obtained.

This adsorptive phenomenon is not thought to have any significance in itself, and is described only to make plain that it has no true relation to specific absorption. The efficiency of the filter block as an adsorptive agent compared with the heterologous suspensions is probably due to the vastly greater area offered

by the pores of the filter, and possibly to the fact that the RA is forced into intimate contact by the atmospheric pressure.

Concerning all the foregoing work a criticism might be made that the assumption that RA is actually taken up for release later is unproven. It might be suggested that it is merely a remnant of the original antiserum which has either escaped washing or has been loosely adsorped to the sensitized mass. Experimental evidence is available. The RA prepared from the first few extractions of a mass invariably consists of H agglutinin only, although the stock antiserum contains ample titres of H and O agglutinins.

An example :

A six-stage extraction was made from *B. typhosus* and its titre was—

H 1 in 128 : O *nil.*

The globulin solution used to prepare the above contained H 1 in 1920 and O 1 in 800.

Similarly a four-stage extraction was made from sensitized *B. paratyphosus B.* The RA titre was—

H 1 in 256 : O *nil.*

Its antiseral solution had titres of—

H 1 in 3200 : O 1 in 640.

Finally the *B. paratyphosus B* antigen was treated with the *typhosus* antiserum and a four-stage extraction made. The result was H *nil* : O *nil.*

It has also been found that RA undergoes a change which modifies the normal mutual attraction of homologous antigen and antibody. The following experiment illustrates the difference :

Released antibody was prepared from a mass, and after the usual heating, was centrifugalized but not decanted from the sensitized deposit. Instead a minimal quantity was withdrawn and examined ; its titre was found to be 64. The tube was then shaken up, the mixture of mass and RA placed in iced water for 5 minutes, after which it was centrifugalized once more. The supernatant RA was decanted and tested. Its titre was still 64, and its volume 2·5 c.c. Some fresh antiseral stock was diluted until it had a titre of 64. To the sensitized mass already used 2·5 c.c. of this diluted stock was added and the tube given 5 minutes in iced water. The tube was then spun without heating or washing and the supernatant collected and titrated. The titre was 2 only.

It is probable that the ease with which RA can be concentrated is largely dependent upon the inability of RA to rejoin a sensitized mass.

Alcoholized Antigens.

Smith and Reagh (1903) first distinguished between flagellar and somatic agglutination, and Beyer and Reagh (1904) showed that the flagellar type of agglutination could be prevented by first exposing the suspension to moderate heat—70° C. Subsequent experience has shown that a higher temperature is desirable, but the essence of these workers' observations is established.

It was shown by White (1927) that treatment of flagellar antigens with ethyl alcohol resulted in the abolition of flagellar agglutination. It is sometimes assumed that the H antigen is totally destroyed by the alcohol. It can be demonstrated that although the agglutinating phase is inhibited, the prior reactions are unimpaired.

The alcoholized antigens used in this work were treated with a large volume of ethyl alcohol for 24 hours at 55° C. The period of action of the alcohol is not critical : prolongation to 72 hours does not change the suspension further. Before resuspending in saline the organisms should be washed once with fresh alcohol ; if this is not done some of the fatty material extracted remains and is precipitated in the aqueous suspension, where it may create a tangled mass of organisms and air bubbles. The mass then floats to the surface during centrifugalization and much of it is lost on attempting to pour the supernatant.

When an alcoholized antigen is treated with its homologous antiserum no visible change occurs, but after washing away the free antiserum in several changes of saline the suspension can be used as an antibody solution and titrated against normal homologous antigen.

It must be stressed that the absorption of antibody thus demonstrated has nothing in common with the non-specific adsorption of RA described earlier because union occurs invariably and at once ; an alcoholized antigen similarly treated with a heterologous antiserum is always inert.

The following record illustrates the specificity of absorption by alcoholized antigens.

Six c.c. of *B. typhosus* H and *B. paratyphosus A* H (Oxford standard suspensions) were alcoholized and brought back to the original volume in saline. Three c.c. of each were treated with the homologous antiserum, and another 3 c.c. with the antiserum of the other organism. The four tubes were allowed two minutes for absorption, then the suspensions were washed three times and resuspended in the original volume of fresh saline. Sensitization and washing were done at low temperature. No agglutination was seen in any tube.

Each suspension was next used as if it were an antiseral solution, and titrated against its own normal Oxford H suspension and also against the normal suspension of the other organism :

Sensitized Antigens.	Titres. With *B. typhosus* H.	Titres. With *B. paratyphosus A* H.
1. Alcoholized *B. typhosus* H with homologous antiserum	6	0
2. The same with *paratyphosus A* antiserum	0	0
3. Alcoholized *B. paratyphosus A* H with homologous antiserum . . .	8	0
4. The same with *typhosus* antiserum .	0	0

The readings show that absorption is specific, and that sufficient antibody is carried by the alcoholized antigen to enable some to be discarded at 56° C., and passed to the normal suspension. Elevation of temperature was not necessary for agglutination.

The above experiment was repeated without heating at 56° C., the whole observation being carried out at room temperature. The time required for visible agglutination was longer, but the titres obtained were the same.

The experiment was further varied. Before the last washing the sensitized alcoholized suspensions were heated for 10 minutes at 56° C. to cause them to shed some antibody if that were possible. The titres obtained were :

	Titres. With *B. typhosus* H.	With *B. paratyphosus A* H.
1. Alcoholized *B. typhosus* H carrying homologous antibody	4	0
2. The same with *paratyphosus A* antibody	0	0
3. Alcoholized *B. paratyphosus A* H carrying homologous antibody	4	0
4. The same with *typhosus* antibody	0	0

The difference of titre in the cold and heated sensitized suspensions was always found, and shows that part of the antibody is released on heating in a manner analogous to the release of antibody by normally agglutinated masses, and also that some is retained by the antigen. It is not detached by heat, but will cross to the agglutinable antigen because of its greater combining power.

In the course of the above experiments a phenomenon has been observed which presents confirmation of the conclusions of Topley, Wilson and Duncan (1935). They sought to show that a specific bond linkage was the cause of the agglutination after specific union had occurred. They used a mixture of two agglutinated organisms, and differentiated them in the floccules by their staining reactions. A comparable result was obtained by Heidelberger and Landsteiner (1923), using haemoglobin with horse serum and antihorse serum. They found that haemoglobin, detectable by its colour, did not precipitate non-specifically.

When an alcoholized antigen carries its homologous antibody the former always remains suspended in the fluid, and is never carried down with the floccules formed when normal suspension is added. It is a commonplace that the end-point tube of a normal agglutination usually shows floccules in a turbid surrounding fluid, whereas the tubes containing stronger concentrations of antibody present floccules lying in a fluid which is almost water clear. In these " carrier " experiments all the tubes are turbid when showing floccules. The following experiment is typical:

A fresh bottle of Oxford H antigen of *B. typhosus* was taken; a portion of known volume was alcoholized, and finally restored to its original volume with saline. This and a suitable amount of normal suspension were each sensitized with *typhosus* antiserum in sufficient excess, washed several times at low temperature, and made up to their original volumes again. They were then used as antiseral solutions, and titrated with the normal antigen from the same bottle. The whole procedure was repeated with *B. paratyphosus A* H. All agglutinated 1 in 8. The description in the table refers to the appearance of the fluid between the floccules in the tubes showing agglutination:

Sensitized antigens.	Dilutions. 1 in 2.	1 in 4.	1 in 8.	1 in 16.
Alcoholized *B. typhosus* H	Turbid	Turbid	Turbid	No agg.
Normal ,,	Clear	Clear	,,	,,
Alcoholized *B. paratyphosus A* H	Turbid	Turbid	,,	,,
Normal ,,	Clear	Clear	Clear	,,

No flocculation was found when each of the sensitized antigens was treated with the normal H antigen of the other organism.

Control tubes of each sensitized alcoholized suspension, with the appropriate amount of additional saline, were placed in the bath. The turbidity of these tubes was compared with that of the reacting tubes. They were found to be indistinguishable. The turbidity of the "no agglutination" tubes was unmistakably greater than that of the control tubes.

Comparison of Yield of Released Antibody.

The amounts of agglutinin released by normally agglutinated and alcoholized suspensions were compared. The method of release is the same. Aliquot parts of a stock suspension were used and the antibody discharged into a standard volume. *Paratyphosus* bacilli *A* and *B* were similarly examined:

	Titre of released antibody.	
	Agglutinated.	Alcoholized.
B. typhosus	32	128
B. paratyphosus A . . .	16	32
„ *B* . . .	16	48

A series of four controls showed that the absorption by the alcoholized antigens was specific, no antibody being taken up by any alcoholized organism when treated with either of the other two antisera.

It is clear that the supposition that ethyl alcohol completely destroys the flagellar antigen is incorrect; the antigen is capable of specific absorption, but cannot carry the reaction to the final stage of agglutination.

Sensitized alcoholized suspensions can absorb and release repeatedly antibody from antiserum as the temperature is raised and lowered, and are therefore suitable for the preparation of RA. In fact they are superior to agglutinated masses, because they release more at each stage and so yield high concentrations with less trouble.

Examination of RA prepared by the alcoholized method has not revealed anything to suggest that it differs from that obtained with agglutinable suspensions. There is some ground for believing that alcoholized suspensions can be used for a greater number of extractions than a normal suspension.

The performance of the alcoholized antigens has a bearing upon the possible truth of Ehrlich's conception of bacterial agglutination, ascribed by him to antibodies of his second order. He considered that two separate and independent reactions occurred. The haptophores determined the specific union, and the zymophore groups reacted to change the stability of the suspension and cause flocculation.

That the haptophore reaction actually exists is evident from the behaviour of the alcoholized antigen when brought into contact with its homologous antibody, and it has been shown that the reaction is as specific as that of a normally agglutinated mass. It is submitted that proof of that phase has been established by direct experiment.

It must be clear that since union alone will not produce agglutination, a second reaction is bound to exist. The question is whether it is specific. It has been

shown that a sensitized alcoholized antigen will not cause any heterologous intact antigen to agglutinate, and that the alcoholized portion does not flocculate in the presence of its agglutinating normal counterpart. These facts may be viewed in the light of the generally accepted belief that the essential change causing flocculation is the denaturation of the immune globulin, resulting in a large change in its iso-electric point. Released antibody, although differing in some respects from its parent antibody, shows no such change, therefore the denaturation must occur in the second phase.

Denaturation cannot spread from an agglutinated antigen to its alcoholized counterpart, so one must infer that there exists on the antigenic site something which cannot be replaced from an external source. Such inferences fall far short of proof, but they suggest that Ehrlich's conception is probably true.

Examination of a Single Large Sample of Released Antibody.

In the preceding record the investigations have been made upon a large number of individual samples, often prepared from different organisms or fractions. It was thought desirable to describe a more detailed study of a single sample of RA in order that the various determinations could be seen in closer relation to each other.

For this purpose 330 c.c. of *B. typhosus* RA were prepared. The stock antiseral solution was the globulin fraction precipitated by 22 per cent sodium sulphate, and washed twice in 22 per cent sulphate solution to remove any significant amount of albumen.

Alcoholized antigen was used and double range titration. The nitrogen determinations were done by Folin-Wu ashing and nesslerization, and read in a Hilger absorptiometer. As the stock of RA accumulated it was frozen solid, and kept in that state until the required volume had been prepared. The contents of the numerous tubes were then pooled and centrifugalized to remove the faint deposit commonly found on thawing after a first freezing. The titrating antigen was the Oxford H suspension.

The following were the results:

A. Globulin solution from which the RA was prepared.
   Titres: H 2560 : O 960.
   Nitrogen content: 33 mg. per cent.
   1 mg. per cent of nitrogen therefore equalled 78 units of agglutination.

B. Titre of the RA: H 128 : O *nil* (or a very doubtful 2).
   Nitrogen content of RA: 1·7 mg. per cent.
   1 mg. per cent of nitrogen = 76 units of agglutination.
   An attempt was made to obtain a protein reading with salicylsulphonic acid, but the amount was too small to determine.

C. A portion of the RA was filtered through a porcelain block and the filtrate examined. The titre was *nil*.
   The remainder of the filtrate was precipitated with 22 per cent sodium sulphate. A minute quantity of deposit was obtained, some of which did not redissolve in saline.
   Titre of the redissolved deposit: *Nil.*
   Nitrogen content „ 1·0 mg. per cent.

D. A portion of the RA was precipitated with 22 per cent sodium sulphate and redissolved in its original volume of saline. The titre of the final solution was 64.

A loss of 50 per cent in titre therefore resulted. The washed insoluble fraction did not show any active agglutinin.

E. 250 c.c. of RA was precipitated with sodium sulphate. The precipitate was collected and redissolved in 16 c.c. of saline, thereby concentrating it 15 times. The insoluble fraction was removed, and the supernatant frozen and thawed to remove any further precipitate. Actually none was formed.

H titre of concentrate . . .	1280
Nitrogen ,, . . .	2·75 mg. per cent.

1 mg. of nitrogen = 465 units of agglutination.

It will be noticed that the activity of the concentrate is in terms of nitrogen six times that of the globulin from which it was derived.

F. Some of the concentrate was passed through a filter block and the filtrate examined.

Titre of filtrate	*Nil.*
Nitrogen ,, ...	1·75 mg. per cent.

The interpretation to be put upon this result is obscure. If the whole of the nitrogen had disappeared on filtration in company with the titre it might be legitimate to assume that one was at last dealing with pure antibody; if none of the nitrogen had been lost it could have been conceded even then that pure antibody was present, the loss of titre being explained by an assumption that the antibody was in a highly active state, which caused the determinant group to disintegrate on coming into contact with the surface of the filter. But neither of these simple beliefs is possible when the whole of the titre and only part of the nitrogen are lost.

One explanation of what is actually found is that two bodies are present—the true antibody and another globulin which has itself no specific activity, but plays a part, not necessarily an essential one, in the process of agglutination.

The RA was prepared from the globulin fraction precipitated by 22 per cent sodium sulphate, and the active and inert fractions of RA are themselves precipitated by sodium sulphate. It is concluded that both fractions must contain a globulin, one of them the actual agglutinin, the other an inert globulin of unknown function. But the apparently inert globulin must have been absorbed during the initial specific absorption, otherwise it could not have appeared in the RA.

It has been shown by Muir and Browning (1906) and by Dean (1917) that non-immune globulin can be utilized, when in the presence of the immune globulin, to form a larger precipitate, and the phenomenon has been termed conglutination. It is possible that the inert globulin shed during the release of antibody is the conglutinating globulin.

Although the preparation of large quantities of RA is tedious, it seems likely that further study of these large volumes offers a prospect of acquiring fresh information concerning the mechanism of agglutination.

SUMMARY.

1. Sensitized bacilli can release antibody at high temperature and take up more at low temperature. The process can be repeated several times.

2. Alcoholized bacilli absorb antibody specifically and release larger amounts than do normal bacilli. The cycle of absorption and release appears to be identical with that of normal bacilli.

3. The released antibody is a globulin, and is accompanied by another globulin which is not specific.

4. Released antibody presents some characters which differentiate it from serum antibody. The change does not amount to denaturation.

5. A method is described for preparing released antibody in comparatively high concentration.

6. Flagellated organisms were used, and the antibody released by them was confined to the flagellar type.

7. It is believed that the behaviour of alcoholized bacilli offers some confirmation of the double specific bond theory.

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THE PREPARATION OF PURE FLAGELLAR ANTIGENS FROM SALMONELLA.

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THE use of H antigens for the investigation of flagellar agglutination is made possible by the fact that the antisera, after absorption of the O antibodies, can be made to react with the flagella alone, although the bacteria are complete organisms possessing both types of antigen.

Various methods have been used to increase the flagellar proportion. Henderson (1932) employed a mechanical method for detaching the flagella from the bacillary bodies. After shaking for an hour the suspension was centrifugalized

for 10 minutes at two or three thousand r.p.m., presumably in the ordinary "fly out" type of centrifuge. The supernatant contained the flagella mixed with some bacillary bodies. The reagents are said to have been water clear and large loose flocculi formed rapidly.

Craigie (1931), in an extensive investigation, used a shaking method also. He found that alcohol on heating at 100° C. for 3 hours did not destroy the H receptor of *B. typhosus*, but changed it to an unsuitable state for agglutination. The release of antibody from alcoholized suspensions (Jenkins, 1946) supports this finding in so far as it relates to the action of alcohol. Craigie considers that the morphological element, the flagellum, is not immunologically altered when broken up into a dispersed physical state, and bases his opinion upon the similarity of the agglutinating and precipitating titres. He failed in several attempts to obtain a specific soluble substance from a pure flagellar suspension, and suggests that flagellar agglutinogen is a compound antigen composed of a non-antigenic substance combined with an antigenic protein. Craigie concluded that the specific substance of flagella cannot be isolated by methods applicable to residual antigens (SSS) (Jenkins, 1921 ; Zinsser, 1921). He found further that exposure of antigen to 100° C. does not affect precipitability.

Recently Seligmann (1945) has prepared saline flagellar extracts by mechanical shaking of living suspensions for 24 hours. The antigen is contained in the supernatant after centrifugalization at 3000 r.p.m. for 2 hours.

In the course of his investigations he ascertained that H antigens when separated from the somata are not destroyed by 2 hours in a boiling water bath. Heating destroys the power to agglutinate, but specific inhibition remains intact and the author relies on it as a proof of specific activity.

Such heated H suspensions were incapable of producing agglutinin or precipitin response, so presumably a flagellar hapten is responsible for the inhibition.

The term H antigen is universally understood, and if reference is to be made to a product containing nothing but the flagellar antigen, it must, to avoid confusion, be designated by some other term. It is proposed that pure flagellar antigens be called FX antigens. Whether they are suspensions or solutions is discussed later.

By the action of dioxan (diethylene dioxide) or of pyridine FX antigens can be prepared from whole suspensions. The strain of *B. typhosus* used for most preparations was H901 which, with the strains "Watson" and Ty 2, were kindly supplied by Dr. Felix. It was found that both reagents destroyed the Vi antigen, but H901 was chosen because its lack of Vi was a theoretical simplification.

Preparation of FX Antigens.

Method 1. *By dioxan.*

A suitable amount of H901 is grown and killed by 0·25 per cent formaldehyde acting for 24 hours at room temperature. Quantities commonly used are 30 c.c. of 10,000 million per c.c. suspension in saline. The culture is harvested and washed twice in saline to remove any medium present in solution. Ten per cent of dioxan is added to the suspension and left in contact for 30 minutes at room temperature ; the flask is given an occasional shake. Next it is centrifugalized for 10 minutes in the angle centrifuge, the supernatant collected and spun twice again for 10 minutes each time.

The angle centrifuge will bring down an ordinary suspension completely in 5 minutes. Dioxan causes a slight translucency of the suspension, probably due to bacillary swelling, and 8 minutes is required to yield a clear supernatant. The translucency is more marked with pyridine. After this treatment no bacillary bodies are present in the supernatant. The product is colourless and crystal clear when viewed in a thickness of 2 cm. A column 8 cm. thick has a faint yellow-green tinge and a barely perceptible opalescence.

The next step is to remove the dioxan. Its B.P. is about 94° C., but it volatilizes at lower temperatures and the fact is utilized in one method of removal.

A pressure filter flask is fitted with a single hole rubber stopper, and through the latter is passed a glass tube of 2 mm. bore reaching the bottom of the flask. The tube should be bent slightly below the stopper so that it reaches the edge of the bottom of the flask if the latter is conical.

Above the stopper the tube should project about 12 cm., and should be given a small kink immediately above the stopper by heating a point and giving the tube a half-turn twist. This prevents the wool being sucked down.

The tube above the kink is then packed as tightly as possible with cotton wool for a length of about 6 cm. The wool offers considerable resistance to the passage of air, and acts as a reducing valve during the subsequent operation.

After noting the volume of the supernatant it is placed in the flask and the latter connected to a vacuum pump. The air bubbles through the solution at reduced pressure and carries the volatilizing dioxan with it. The extent of the removal is judged by examining the volume. When the fluid is reduced to two-thirds it is deemed free of dioxan. Experiment showed that a small proportion of dioxan does not interfere with the flagellar reaction.

The above procedure can be used to concentrate the antigen further, but if a considerable degree of concentration is desired it is advisable to use half the normal strength of saline for the original bacillary suspension; otherwise the salt concentration may be inconveniently high. For the same reason distilled water should be used if it is necessary to increase the volume of the solution when placing it in the flask. To reduce the volume to half requires 3 to 4 hours' bubbling, according to the rate of flow. At the conclusion the colour of the antigen is slightly deeper, but the opalescence is not noticeably increased.

The antigen can be further concentrated as it will not pass a candle. After all the supernatant has been filtered about 50 c.c. of saline is passed and the candle drained for a few seconds by further suction. It is then taken out and turned, delivery nozzle uppermost. With a pipette the interior is filled with saline and a short piece of rubber pressure tubing connected to the nozzle. A record syringe is filled with saline and its nozzle connected to the other end of the tubing. The whole contrivance is then held over a beaker and the plunger pressed down hard. The saline is forced at high pressure through the candle and carries much of the antigen with it.

The " reverse " suspension is active and almost as transparent as the original. A few flakes present can be broken up by shaking. The greenish tinge passes with the filtrate. It is essential to remove the solvent before proceeding to concentrate by the filter method.

Care should be taken that an antigenic solution is not completely evaporated overnight as the antigen does not stand drying.

The advised strength of 10 per cent dioxan is the result of various trials.

Five per cent acting overnight gives a rather weaker antigen, and the washed bacillary bodies still react weakly to H antiserum. Fifty per cent dioxan destroys the flagellar antigen completely.

Method 2. *By pyridine.*

The alkalinity of pyridine makes it unsuitable for use alone, so a 70 per cent solution in saline is brought to pH 7·0 with HCl. The neutralized mixture is diluted with saline to make a 40 per cent solution and is the stock solution. It appears to keep indefinitely.

Five per cent. of the stock solution is added to a suspension and allowed to act overnight. Further procedure is as for dioxan. As the B.P. of pyridine is 114° C., several hours are required to remove it, and it has been found expedient to bubble it with an equal volume of distilled water overnight. The HCl is apparently removed at the same time, since the pH of the final solution is still 7·0. The absence of odour is assumed to indicate that removal is complete. As with dioxan, a small proportion does not interfere with the immune reaction. Reversed filtration can also be used. The somatic antigens are left intact by both solvents.

The appearance of the flocculi formed when the antigen reacts with its antibody is distinctive. The reaction is carried out in the 55° C. bath. A few small transparent flakes resembling mucus first appear, and are visible only on close inspection against a black background. Gradually they coalesce to form one or two masses much larger than those seen in H agglutination. They are then more readily seen as iron grey clouds, and never at any stage assume the cotton-wool appearance of an H antigen. It is possible that the latter owes its character to the opaque bacillary bodies which necessarily accompany an H agglutination.

Little information is available concerning the amount of FX antigen obtained relative to the total bacillary suspension before extraction. From some very indirect references based upon thepro portion of antibody bound, it is probable that there is an appreciable loss.

First experience with FX antigen and its total yield of precipitate compared with the massive quantity of H flocculi obtainable from the suspension from which it was prepared engenders disappointment at the smallness of the yield. But on reflection it will be seen that there is no common basis for comparison. The adhesive factor in an H agglutination is the binding together of the flagella, which are in turn attached to the somata, which are some distance apart.

Specificity.

Initial titration is done by the alpha technique because it enables comparison of the strength of various samples to be made. In this method a constant dilution of antiserum is titrated against a falling concentration of antigen. The standard of comparison is the agglutination obtained with Oxford H antiserum, and each sample of antigen was diluted sufficiently to give an endpoint of 256 with 1 in 40 antiserum after 2 hours in the bath at 55° C. The alpha technique obviates the risk of dismissing a sample as inert, when in fact it is excessively strong in relation to the initial dilution of serum.

To secure this standard the antigens as prepared need dilution 2 to 32 times. The yield from *B. typhosus*, *B. stanley*, or *B. muenchen* is satisfactory. *B. stanley*

is at least as rich a source of *d* antigen as *B. typhosus*, and probably better than *B. muenchen*. The last is the source of choice if it is desired to obtain an FX *d* antigen which shall be free from all suspicion of traces of *B. typhosus* somatic antigens. The precaution is academic, as no evidence has ever been found to indicate that any somatic antigen was present.

The specificity was examined by preparing extracts of the undermentioned organisms and titrating them against the relevant antisera:

Antigens.	Antisera.		
	Typhosus.	*Paratyphosus.*	
		A	B	C
B. typhosus H901	256	0	0	0
,, Watson	128	0	0	0
,, *Ty* 2	128	0	0	0
,, Lab. strain	256	0	0	0
B. stanley	256	0	0	0
B. muenchen	128	0	0	0
B. paratyphosus A	0	128	0	0
,, *B*	0	0	128	0
,, *C*	0	0	0	64

No agglutination was obtained with O sera.

All the positive reactions showed the same diaphanous grey clouds. It was concluded that the specificity of the FX antigens is in no way inferior to that of their corresponding H antigens.

Nature of FX Antigens.

FX has been kept at 4° C. for periods up to ten months. After about 20 days a very thin turbidity develops which can be removed by the centrifuge. The supernatant alone is antigenic and does not lose titre. Whether the antigen is in solution or is particulate is uncertain. Prolonged centrifugalization at 3000 r.p.m. does little to diminish the activity of the supernatant. Reversed flow filtration suggests a particulate nature. It is probably a suspension in a fine state of division. FX is destroyed in 10 minutes at 60° C., and instantly on boiling. Alternate freezing and thawing has no effect upon it.

The biuret reaction is clearly positive. The xanthoproteic reaction is very faint, but can be detected by comparison with a blank. No reaction was obtainable with Millon's reagent. Every attempt to stain for flagella has failed; only an amorphous deposit has been seen.

The salicylsulphonic acid test for protein is useless. A sample with an alpha titre of 64 showed a barely recognizable turbidity much too weak for quantitative measurement.

Suspensions vary in their power of reacting with Nessler's reagent in the cold, and they therefore cannot be used for N determinations in that state. Reversed flow filtration secures a solution which is free from these reacting bodies and can be used for antigenic N measurement. Such preparations are the purest form of FX antigen, and can be further concentrated in the bubbling flask.

Half saturation with ammonium sulphate gives a precipitate which can be redissolved in saline ; a small precipitate then remains and is antigenically inert on resuspending in saline. The fraction which dissolves is still active.

Attempts to extract a specific polysaccharide all failed.

Seligmann's findings were readily confirmed. The antigen was *B. typhosus* FX used undiluted. The serum range was 1 in 10 to 1 in 320.

Experiment 1.

Unheated FX antigen with the agglutinating serum. Result : Agglutination to 1 in 160.

Experiment 2.

The antigen was boiled briskly for 10 seconds, cooled, and titrated as before. Result : No agglutination.

Experiment 3.

One standard volume of the boiled antigen was mixed with the same dilutions of serum and placed in the 55° C. bath for 10 minutes. Then a standard volume of unheated antigen was added to each tube and the tubes replaced in the bath for 3 hours.

Result : Agglutination in 1 in 10 only. This was ascribed to excess of antibody after the boiled antigen had taken up all it could hold. The readings conform to the accepted belief that an antigen can absorb up to 8 times the minimal amount of antibody needed to produce recognizable reaction.

Experiment 4.

One volume of heated antigen and one of unheated were mixed in each tube and the appropriate serum dilutions then added. The tubes were read after 3 hours in the bath. Result : Agglutination 1 in 80.

The last titration shows that the heated antigen, unlike agglutinoid, has no preferential affinity for the antibody, but competes on equal terms with the normal antigen.

The absorption experiments were repeated with FX antigens prepared from the four *B. typhosus* strains used in this work and from *B. muenchen* and *B. stanley*. They were first diluted to bring them to the same endpoint with the standard serum, and then tested for absorption (inhibition) in all their possible permutations. It was found that they all gave identical results, and nothing was seen to indicate that their *d* antigens were not identical.

Seligmann's work demonstrates that the flagellar antigens have at least two reacting groups, which confirms the results obtained with alcoholized antigens. The apparent total destruction of the flagellar antigen when it is heated, whilst still attached to its soma, offers the obvious but vague assumption that the flagellar protein is linked with a heat-labile somatic substance, and in that state hares its denaturation. This would imply that FX may be the flagellar hapten.

SUMMARY.

1. A flagellar antigen has been extracted from several Salmonella.

2. Dioxan or pyridine is used for extraction and methods are described for removal of the solvent.

3. The antigen is pure in the sense that it contains no somatic antigen.
4. It is a protein and can be concentrated by methods described.
5. Its specificity is equal to that of its H suspension.
6. Heat destroys its zymophore group, but leaves the haptophore group intact.
7. It is possible that the FX antigen is the flagellar hapten.

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A STUDY OF THE PROZONES OF THE SALMONELLA.

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THE prozone phenomenon in bacterial agglutination is usually considered to be related to the question of optimum proportions. That the O.P. ratio has an important role in prozone production is undoubted, but it is not so clear that the whole explanation lies there.

The present work on the prozone is based on the premise that it is an integral part of the immune reaction, and ranks equally with the visible floccules as a manifestation of the reaction.

The prozone was observed in the agglutination of bacteria almost as soon as specific agglutination was first described. Ehrlich attributed it to partial agglutinins which he named agglutinoids, and Streng (1909) showed that in the case of some organisms agglutinoid could be prepared from agglutinin by moderate heating. His results were confirmed by Shibley (1929). The temperature range between agglutinoid formation and complete destruction is apparently narrow. Shibley found that in the case of *B. dysenteriae shiga* washing the organism after sensitization with agglutinoid and then resuspending in saline did not cause agglutination, and that the organism was still incapable of agglutination in the presence of added normal agglutinin. On the other hand, Jones and Orcutt (1934) state that washing of an organism in its prozone range of combination enables flocculation to occur, and they attribute the change to removal of an inhibiting body. The ground for this inference is not clear.

Duncan (1937) failed to find a prozone with flagellated intestinal bacteria, and the results obtained in this work confirm his findings to a certain extent.

The present investigation started with an attempt to produce a flagellar prozone with *B. typhosus* agglutination. It was found impossible to form agglutinoid by heating.

Heuer (1922) and Da Costa (1929) state categorically that prozone can always be demonstrated when very thin suspensions are used. The statement was tested, and partial confirmation obtained.

The experiments are discussed here as they are set forth, but it may be well to make clear the meaning of the term "tubal order of agglutination." The range of dilutions in the 55° C. bath are inspected every 5 minutes for 2 hours. The dilutions are set out in a line, and supposing that 8 dilutions are used, starting with 1 in 4, the range written down will be—

4	8	16	32	64	128	256	512

The first tube to show visible agglutination on inspection with a hand lens is called No. 1 in the tubal order, and the figure is written beneath the appropriate dilution number. Thus if the agglutination started in the first tube, No. 1 would be below the 1 in 4 dilution and the other numbers would follow in serial order. For brevity such a result is called "serial."

But if the 1 in 32 dilution first showed floccules the figure 1 is written beneath that dilution, and the second and further tubes to do so beneath their dilutions. If no agglutination is visible in a tube at the end of the period of recording it is marked with an 0. The following suppositious reading will illustrate the latter:

Dilutions:	4	8	16	32	64	128	256	512
Tubal Order:	0	0	4	3	1	2	5	–

This means that there was a complete prozone in the first two dilutions; the first tube to agglutinate was the 1 in 64 dilution, followed by 128, 32, 16, and 256 in that order.

Experiment A.

Suspension: Oxford H antigen diluted 1 in 3.
Antiserum: Burroughs Wellcome, titre 51,200 to H when using normal strength Oxford H antigen.

A range of 12 tubes was put up, starting with 1 in 2 dilution. The tubal order of agglutination was:

12	8	3	1	2	4	5	6	7	9	10	11

It will be seen that there is no actual prozone, but there is a decided prozonal lag.

The experiment was repeated with another sample of B.W. antiserum which had a titre of 64,000 for H. The tubal order was:

11	7	5	1	2	3	4	6	8	9	10	12

The two sets of results are in good agreement. In the second example the "best" tube had a seral concentration of 4000 times that of the endpoint tube. Disparity of proportion could hardly be carried much further.

Experiment B.

In this the Oxford H serum was used and titrated with Oxford H antigen of *B. typhosus* diluted 1 in 4. The endpoint was 2048 visible with a hand lens.

Tubal Order:	10	8	4	1	2	3	5	6	7	9	11

The titration was repeated with a slightly stronger antigen, obtained by diluting 1 in 3. The result showed the slight variation to be expected.

Tubal Order: 7 6 3 1 2 4 5 8 9 10 11

The B.W. sera give results in good agreement with each other, and the same is true between the two Oxford sera, but there is wide discrepancy between the B.W. sera on the one hand and the Oxford sera on the other. In the former the ratio of concentration between the "best" tube and the endpoint is 4000 to 1 and 3200 to 1, whereas with the Oxford it is 128 to 1.

If the B.W. sera are used as a base for calculation there should be no prozonal lag in the Oxford titration. If, on the other hand, the Oxford serum is taken as the base, the best tube of the B.W. series should have been the seventh or eighth tube. It is evident that the prozonal lag, which is presumed to be an incipient prozone, cannot be wholly explained by the disparity in the proportion of antigen to antibody.

Experiment C.

High titre released antibody (RA) (Jenkins, 1946*a*) was tested with Oxford *B. typhosus* H antigen diluted 1 in 4. Tubal order was serial.

The endpoint of the RA was 1024, coarse floccules, and the result is therefore comparable with that of Experiment B.

If a prozonal factor is present in serum it is absent in RA. It has been observed constantly that the velocity of the RA reaction is definitely greater than that of antiserum. It is probable that the RA, during its period of union with the sensitized mass, has undergone some preparatory change which shortens the time required to reach the final stage of visible agglutination.

Various explanations might be put forward concerning the total absence of prozonal lag. If the RA is prepared from an antiserum containing small amounts of agglutinoid and the latter is more firmly bound to the antigen it might be left on the antigen when the RA is shed. The result would be an RA solution entirely free of agglutinoid.

Experiment D.

In contradistinction to the H antigens, the FX antigens (Jenkins, 1946*b*) can be made to develop a definite prozone. A specimen of *B. typhosus* FX was titrated with Oxford H serum, standard titre 320.

Antigen dilutions.	Antiserum dilutions.							
	4	8	16	32	64	128	256	512
Not diluted	A	A	A	A	A	A	O	O
Tubal order	Serial							
Diluted 1 in 2	A	A	A	A	A	A	A	O
Tubal order	Serial							
Diluted 1 in 4	A	A	A	A	A	A	A	O
Tubal order	4	1	1	3	5	6	7	
Diluted 1 in 8	O	O	A	A	A	A	A	A
Tubal order	0	0	3	1	2	4	5	6

A = Agglutination.

It will be observed that the concentration of antibody in the tube of most rapid flocculation is only 16 times the endpoint concentration, and the fact might be adduced as evidence that the FX antigen is a solution, and the ease with which a zone appears as an indication of a precipitin reaction.

The above findings were confirmed by two observations with the alpha technique, in which a constant dilution of antiserum is titrated against a falling concentration of antigen. An FX antigen of H901 was titrated against Oxford H serum, dilution 1 in 128. The antigenic dilutions started at 1 in 2. The tubal order was :

7 5 4 1 2 2 6.

As a source of *g* antigen the Derby bacillus (*fg*) is much richer than *B. enteriditis gaertner* (*gom*), and prozone is correspondingly more pronounced. FX antigens were prepared from approximately equal suspensions, and alpha titrated with *gaertner* H serum 1 in 40. The antigen started at 1 in 2.

FX antigens.	Endpoint.	Tubal order.
B. enteriditis gaertner .	32 .	4 2 1 3 5
B. derby	256 .	0 0 0 0 3 1 2 4

If there is anything in the two-stage enzyme conception of agglutination the absence of lag with RA is not inconsistent, because the excessive substrate would make its influence felt in the earlier phase of the reaction with fresh immune serum ; once that phase of activity is passed and the hypothetical second enzyme begins to operate, the immune globulin ceases to be the substrate and its concentration would have no influence upon the activity of the second anzyme.

The Prozone of Somatic Antigens.

In the preceding investigations the prozone was determined either by disparity of antigen and antibody or by changes in the antibody, natural or induced, It can be shown that in the case of the somatic antigens prozone is also determined by the state of the antigen, and that agglutinoids are unlikely to have anything to do with the results obtained.

A culture of H901 was killed with formalin, then treated with 40 per cent neutralized pyridine for 24 hours at room temperature. After washing the suspension was diluted to a weaker concentration than that normally used for agglutination.

It is necessary that different suspensions should be approximately equal in strength, and the following method was found practicable. The suspension was diluted to a concentration approximating to that used for normal agglutination, then portions were taken, and diluting by doubling until a suspension was obtained which was barely visible when inspected under a strong light against a black background. The desired concentration is six times that of the extreme dilution. The tubes to be used in the agglutination should be used for the opacity estimation. Where possible the antisera should have an endpoint of about 250 with an Oxford standard antigen, and the seral dilutions should start at 1 in 2. For the sake of brevity 40 per cent neutralized pyridine somatic antigens will be termed P40 antigens.

A portion of the P40 H901 was heated for 2 hours at 100° C., then the heated and unheated standardized antigens were titrated with Oxford *typhosus* O serum. The tubal orders were :

Heated : Serial						
Unheated : 0	0	4	3	2	1	5

A repetition with Watson, Ty 2, and the laboratory strain showed a similar prozone with their P40 antigens. There was no evidence that the Vi antigen had any influence.

In order to ascertain if the pyridine acted on the polysaccharide, the suspensions were heated for 2 hours at 100° C. and the pyridine then added. All the suspensions agglutinated in serial order.

It was observed that for the development of a good prozone the temperature should be about 57° C. At 50° C. the prozone is still present, but not as marked. The tubes should be inspected at frequent intervals in order to determine the tubal order, since in some instances there may be no actual prozone, but only a prozonal lag.

It was concluded that the pyridine acted upon a component of the compound somatic antigen which was not the hapten.

In reviewing the findings it was realized that one possibility had not been examined. The broad suggestion was that the prozone-producing factor might not be conjugated to any antigen, but might be an independent body, in which case it might normally reside in the somata or the flagella, or be distributed in both. A short inquiry decided the point. Parenthetically it should be said that FX antigen can be prepared equally well from 2 or 20 per cent pyridine. The latter is not recommended owing to the trouble in removing so large a concentration later. Only 20 or more per cent produces the somatic prozone.

FX antigen prepared from 20 per cent pyridine acting on H901 was alpha titrated and its endpoint noted. A similar FX by dioxan was titrated and both were adjusted by dilution to the same endpoint. The titrations were then repeated and the prozones compared. It was found that the pyridinized preparation had no increased tendency to prozone. The pyridine effect is therefore peculiar to the somatic antigens.

In the next experiment a heated O suspension of Ty 2 was mixed with its 20 per cent pyridinized FX antigen and the mixture titrated with O serum. No prozone was found, confirming that the prozone factor did not reside in the flagellar fraction.

Finally P40 *B. muenchen* was added to the somatic fraction of a 2 per cent pyridinized *B. typhosus* and the mixture titrated with typhosus O serum. No prozone resulted. The experiment shows that the factor cannot cross to an antigen-antibody system not so treated.

The possibility that the polysaccharide and the unknown exist as independent entities is further discounted by the fact that if all the O antibody is removed by heated O antigen, no reaction can be obtained on subsequent titration with P40 antigen. The two antibody components disappear together and presumably react with the same antigen. It seems a fair inference that the antigen in nature is compound, and that the antibody molecule carries determinant groups for both fractions.

The compound nature of the somatic antigens is generally accepted. Morgan and Partridge (1942) separated a polysaccharide, a phospholipin, and a protein from *B. typhosus* somata. Landsteiner (1945) considered that species-specificity was due to the protein fraction, at least in so far as the Salmonella were concerned.

The prozones of P40 antigens are species-specific, and the following range of titrations illustrate the phenomenon.

The antisera of *B. typhosus* O and *B. typhi murium* O were the Oxford standards. That for *B. pullorum* was kindly supplied by the Institute of Animal Pathology, Cambridge, and that for *B. enteriditis gaertner* by the Oxford Laboratory. The O serum of *B. paratyphosus B* was obtained by absorbing the H from Burroughs Wellcome whole antiserum.

P40 preparations of two groups of organisms were taken:

Somatic structure, IV, V, XII.	IX, XII.
B. paratyphosus B.	*B. typhosus.*
B. typhi murium.	*B. enteriditis gaertner.*
B. stanley.	*B. pullorum.*

All the seral dilutions started 1 in 2. Owing to scarcity of serum it was necessary to dilute the stock of *gaertner* and *pullorum* sera, so the endpoints are lower than usual and therefore affect the significance of the dilutions.

Antigens.	Antisera. Tubal Order, *typhosus*, E.P. 256.
Set 1.	
B. typhosus	0 0 0 4 1 2 3 5
B. enteriditis gaertner	Serial
B. pullorum	,,
Set 2	With *enteriditis gaert.*, E.P. 128.
B. typhosus	Serial
B. enteriditis gaertner	0 1 2 3 4 5
B. pullorum	Serial
Set 3	With *pullorum*, E.P. 64
B. typhosus	Serial
B. enteriditis gaertner	,,
B. pullorum	2 1 3 4 5
Set 4	With *paratyphosus B*, E.P. 256
B. paratyphosus B	0 7 5 4 1 2 3 6
B. typhi murium	Serial
B. stanley	,,
Set 5	With *typhi murium*, E.P. 256
B. paratyphosus B	7 4 2 1 3 5 6
B. typhi murium	4 2 1 3 5 6 7 8
B. stanley	Serial

The prozones of the *B. typhosus* group are clearly defined, but the other group indicates that the somatic antigens of *B. typhi murium* and *B. paratyphosus B* are more closely related. The results here suggest that *B. typhi murium* has a

somatic antigen also possessed by *B. paratyphosus B*, whilst the latter organism has a single second factor. The greater specificity of proteins in general compared to polysaccharides suggest, that the second factor is a protein. Species-specificity would then be the normal expectation, and only in an occasional instance would two organisms be found to possess a common protein. In this connection it may be recalled that Morgan and Partridge (1942) found by anaphylactic experiment that a protein of *B. typhosus* resembles closely one isolated from *B. dysenteriae shiga*. The P40 antigen elicited, by agglutination, confirmation of their findings.

Owing to the extreme disproportion between *B. typhosus* antigen and its *shiga* antibody an alpha-beta titration was essential: this, a falling series of serum concentration against rising antigen concentration. This technique is rapid as a means of detecting a zone of activity when both constituents are of unknown and widely differing strengths.

Serum and antigen both started at 1 in 2. The initial strength of the suspension was 4 times that used for normal agglutination. Oxford standard sera, titre 250, were used throughout. The controls were also titrated by the alpha-beta technique.

	Dilutions.							
Shiga serum . . .	2	4	8	16	32	64	128	256
P40 *B. typhosus* . .	256	128	64	32	16	8	4	2
Reaction	A	A	A	A	A	O	O	O

A = Agglutination.

Alpha-beta controls:

Flexner polyvalent serum and P40 *B. typhosus* . .	No reaction.
Shiga serum and heated P40 *B. typhosus* . . .	,,
,, ,, P40 *B. paratyphosus B* . . .	,,
,, ,, P40 *B. muenchen*	,,
,, ,, P40 *B. enteriditis gaertner* . .	,,

The positive reaction is regarded as specific as the serum reacts at 1 in 32, and in the strongest serum tube the antigen is in a dilution four times beyond the dilution of faintest visibility. If non-specific agglutination were the explanation the suspension must contain a non-specific antigen far beyond reasonable probability.

The results as a whole show that *B. typhosus* contains a large concentration of a somatic protein which is closely related to an antigen in *B. dysenteriae shiga*, and that the antibody response to the same protein when it is a part of Shiga's bacillus is relatively weak.

The P40 antigens are probably protein and are in the main species-specific. The species agglutination with a prozone is due to the reacting protein, and the serial agglutinations of other members of the same Kauffmann-White group to their common polysaccharide.

It is submitted that the study of prozones is capable of making a contribution to the elucidation of the antigenic structure of bacteria.

SUMMARY.

1. H antigens give a weak indication of prozonal lag.

2. FX antigens develop a prozone more readily than the H antigens, but there is nothing to indicate that the difference is due to any chemical change.

3. Released antibody cannot be made to develop a prozone or prozonal lag. A possible explanation is given.

4. Forty per cent pyridine removes the flagellar antigens from Salmonella, but leaves the somatic antigens in their compound state.

5. The pyridine acts upon the second component, and so changes the complex that prozones are readily produced.

6. The pyridine somatic antigen is species-specific, and will distinguish between most members of a Kauffmann-White group.

7. There is reason to believe that the second component is a protein.

8. *B. typhosus* and *B. dysenteriae shiga* have a common somatic protein which can be demonstrated by cross-agglutination.

9. Dioxan, which removes flagellar antigen equally well, does not influence the somatic complex to produce prozone.

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SURVEY OF PAPERS.

KENNAWAY AND DAFF find that the ascorbic acid content of the liver of mice receiving an ordinary diet falls on a vitamin B-free diet and can be restored to the same or a higher level by a supplement of yeast. Riboflavin and methionine have a similar but not so powerful supplementary action; while milk or skim milk has the same effect as yeast in C57 male mice (p. 63).

WILLIAMS AND HARPER have tested 152 strains of staphylococci for coagulase production by 11 tube methods and the slide method, and for haemolysin production by three different methods. Of 100 coagulase-positive strains 93 produced α-haemolysin and 2 showed only β-haemolysin, whereas none of the coagulase-negative strains produced haemolysin (p. 72).

BAWDEN AND PIRIE have previously shown that in plants infected with tobacco mosaic virus only a part of the virus is contained in the sap, and that at least an equal amount is retained in the fibrous residues. They now find that the fibrous residue contains more virus than can be set free by grinding, and that an enzyme preparation from the alimentary tract of snails is more effective than trypsin in bringing this virus into solution (p. 81).

SELBIE AND HEBBERT find that the curative effect of a single dose of neoarsphenamine in rabbit syphilis is not appreciably increased by the addition of a short course of penicillin, although the two drugs have a synergic action in the early stages of treatment (p. 91).

ARMSTRONG AND BONSER have retested a strain of mice which 6 years previously had been found to be particularly susceptible to carcinogenic agents applied to the skin. Painting with 3 : 4-benzpyrene now shows that the average time of appearance of warts has lengthened considerably, whereas the interval between the formation of warts and the subsequent development of malignancy is reduced (p. 97).

MURRAY has examined 1038 bloods for Rh antigens and gives a statistical analysis of his results, which indicates that the spacing of the loci of the Rh genes on the chromosome is unequal. He predicts the reactions of further possible antigens caused by mutant genes (p. 102).

JENKINS shows that sensitized bacilli can release antibody at high temperature and take up more at low temperature. The released antibody is a globulin, and has some characters which differentiate it from serum antibody (p. 111).

JENKINS has used dioxan or pyridine for the extraction of flagellar antigens from several organisms of the Salmonella group. The extracted antigen contains no somatic antigen and its specificity is equal to that of the original H suspension. It is a protein and heat destroys its zymophore group, leaving the haptophore group intact (p. 121).

JENKINS finds that extracted H antigens develop a prozone more readily than the original H antigens, while released antibody cannot be made to develop a prozone or prozonal lag. Pyridine removes flagellar antigens, leaving the somatic antigens in their compound state, but acts upon the second component and so changes the complex that prozones are readily produced (p. 127).